The Epidemiology of *Clostridium difficile* in a Geriatric Unit

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CONTENTS

DECLARATION

The author performed the investigations and procedures described in this thesis unless indicated otherwise.

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ABSTRACT

C. difficile is the major identifiable cause of antibiotic-associated diarrhoea in the UK. The association of *C. difficile* with the use of antibiotics is well established. A number of other host and bacterial factors are likely to play a role in the development and the severity of *C. difficile* associated disease.

Three hundred and ninety patients between 62 and 101 years of age admitted to a geriatric unit in The Royal Victoria Hospital (RVH), Edinburgh were investigated for the presence of *C. difficile*. *C. difficile* was cultured from 100 (26%) patients, on pre-reduced cycloserine-cefoxitin egg-yolk agar. Toxin(s) were detected in the faeces of 34 of these patients with the Techlab™ ELISA test kit for the detection of *C. difficile* toxins A and/or B. Toxin(s) were detected in a further 18 patients from whom no *C. difficile* was detected in culture.

A number of possible risk factors associated with *C. difficile* disease, and relating to medication, antibiotic use and underlying disease were investigated by logistic regression modelling. A two-step predictive model for *C. difficile* disease was hypothesised. In this two-step disease model, patients made a transition from *C. difficile* negative (Cdc-) to *C. difficile* culture positive (Cdc+) and in some cases a further transition from Cdc+ to *C. difficile* culture and toxin positive (Cdt+) was made. The logistic regression modelling found that the factors significantly associated with the transition from Cdc- to Cdc+ were the origin of the admission (another hospital, nursing home or the community) and the use of ceftriaxone (a third generation cephalosporin). Amoxycillin and cephalosporins (other than ceftriaxone) were significantly associated with the transition from Cdc+ to Cdt+. Statistical analysis also showed that Cdt+ patients were significantly older than the Cdc+

patients. These findings support the proposed two step model for infection and indicate that different risk factors are significant in each of the two steps.

The variation and immuno-reactivity of the surface (S-) layer proteins from 24 strains of *C. difficile* were investigated. The S-layer proteins extracted from *C. difficile* by treatment with guanidine hydrochloride were visualised on SDS-PAGE and analysed using Phoretix^{TM} gel analysis software. The two S-layer proteins were shown to be highly variable in molecular mass between isolates of different serotype and ribotype. Western blot analysis showed that there was cross-reactivity of the heavier protein between strains, however there was much less cross-reactivity with the lighter protein which appeared to be strain specific. The variation in molecular mass of the S-layer proteins was exploited as the basis of a novel typing method for *C. difficile*. Isolates from patients in RVH were designated a four-digit "S-type" number based on their S-layer protein profile. A total of eight S-types were identified, and one type, toxigenic S-type 5236 accounted for 73% of all clinical isolates. No statistical association was evident between the S-type(s) which colonised patients and the number of episodes of diarrhoea or the duration of illness.

A selection of 50 clinical isolates were also typed using PCR ribotyping methods, this revealed ten ribotypes and a good correlation between the two typing methods. The endemic S-type "5236" correlated to PCR ribotype 1 which is the most frequently isolated PCR ribotype in England and Wales.

PUBLICATIONS

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ABBREVIATIONS

AAD	Antibiotic-associated diarrhoea
AIDS	Acquired immune deficiency syndrome
AP-PCR	Arbitrarily primed-polymerase chain reaction
ARU	Anaerobe Reference Unit
CCEY	Cycloserine-cefoxitin egg-yolk agar
CCFA	Cycloserine cefoxitin-fructose agar
CDAD	*Clostridium difficile*-associated diarrhoea
Cdc-	*Clostridium difficile* culture and toxin negative
Cdc+	*Clostridium difficile* culture positive
Cdt+	*Clostridium difficile* culture and toxin positive
CGRP	Calcitonin gene-related peptide
CMB	Cooked meat broth
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
GTP	Guanosine 5'-triphosphate
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin gamma
IM	Intra-muscular
IV	Intravenous

kDa	kilo Dalton
MIP-2	Macrophage inflammatory protein-2
MPRL	Microbial pathogenicity research laboratory
NG/PEG	Nasogastric/percutaneous endoscope gastrostomy
PAGE	Polyacrylamide gel electrophoresis
PaLoc	Pathogenicity Locus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMC	Pseudomembranous colitis
PMS	Pyrolysis mass spectrometry
PPY	Proteose peptone yeast
RAPD	Random amplified polymorphic DNA
REA	Restriction enzyme analysis
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
RS-PCR	Ribospacer PCR
RVH	Royal Victoria hospital
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLP	Surface-layer protein
SP	Substance P
TBS	Tris buffered saline
TNF-α	Tumour necrosis factor-alpha
UV	Ultra-violet

CHAPTER ONE

INTRODUCTION

1.1. *Clostridium difficile*, a description of the organism

Clostridium difficile is a strictly anaerobic, motile, gram-positive bacillus of 3-5μm in length with sub-terminal spores. On blood agar, typical *C. difficile* colonies are opaque, greyish in colour and have a pitted appearance with an irregular edge (Hafiz and Oakley, 1976). Most colonies are non-haemolytic, however some strains do produce alpha-type haemolysis on blood agar and sporulation is distinct on agar cultures that have reached stationary phase (Brazier and Borriello, 2000). *C. difficile* can be distinguished from other *Clostridium* spp. by biochemical profile and the toxins which are produced.

1.2. The history of *C. difficile* and associated disease

1.2.1. The history of *C. difficile*

In 1935 Hall and O'Toole identified a spore-forming, motile, obligately anaerobic bacillus from the intestinal flora of new-born infants. Due to the difficulty associated with the isolation and study of the organism, the bacterium was named *Bacillus difficilis*; this organism is now known as *Clostridium difficile*.

Further investigations were carried out in 1937, when Snyder isolated 18 strains of *C. difficile* from the faeces of babies under one year of age. Snyder's investigations determined that despite similarities in the morphology and culture characteristics, some strains of "*Bacillus difficilis*" isolated from the faeces of babies were pathogenic in guinea pigs and rabbits. Snyder (1937), raised rabbit antiserum to a pathogenic strain of *C. difficile* and experiments demonstrated that all pathogenic and

non-pathogenic strains agglutinated at varying degrees with the antiserum. This suggested that there were antigenic similarities between strains allowing agglutination, but also antigenic differences which affected the level of agglutination between strains.

Following Snyder's work in the late 1930s, there was little interest in *C. difficile*. However, in the late 1970s, *C. difficile* provoked intense interest, when it was identified as the organism commonly associated with pseudomembranous colitis (PMC) and antibiotic-associated diarrhoea (AAD), cases of which had soared with the use of clindamycin.

1.2.2. *C. difficile*-associated disease (CDAD)

Hall and O'Toole (1935), detected toxins from *C. difficile* and postulated that the organism could be pathogenic. *C. difficile* is now well documented as the major identifiable cause of AAD and PMC (Bartlett, 1994; Kelly et al, 1994a).

Diarrhoea is well associated with antibiotic use and reports of PMC after treatment with antibiotics were made in the 1950s. Reiner et al (1952), made an association between diarrhoea and PMC, and the use of aureomycin and chloramphenicol. PMC was also associated with the use of lincomycin, it was hypothesised that the gut flora could be affected by lincomycin use, and anticipated that the closely related drug clindamycin would also be associated with PMC (Scott et al, 1973).

Tedesco et al (1974), made a strong association between clindamycin use and the development of PMC; a prospective study showed a 21% incidence of AAD and a 10% incidence of PMC in a group of 200 patients who had been administered clindamycin. However, no microbiological cause for the disease was determined.

Larson et al (1977), suggested that the aetiology of PMC could be of bacterial origin after a toxin was detected in the faecal suspension from a 12-year old girl who developed PMC following treatment with penicillin. The faecal suspensions from the patient demonstrated a toxic effect on several cell lines. Subsequently faecal specimens from six patients with PMC were investigated for a similar effect. The presence of a toxic factor in five of the six patients with PMC was demonstrated. The study could detect no viral cause and the investigators suggested that the toxin could be of bacterial origin.

Rifkin et al (1977), implicated *Clostridium* spp. as the aetiological cause of PMC, when it was shown that the faecal filtrate from patients with PMC caused death in hamsters, and that the pre-incubation of the faecal extracts with gas-gangrene antitoxin prevented death in hamsters. Further studies showed that the faecal filtrates were cytopathic to *in vitro* cell lines, but that the effect was neutralised by *C. sordellii* antitoxin. Rifkin et al (1977), concluded that *C. sordellii* was the cause of PMC.

In the late 1970s Bartlett et al (1978a), were investigating the aetiology of PMC. They also showed that the faecal filtrates from patients with PMC and with clindamycin -associated diarrhoea could produce a cytopathic effect *in vitro* and that this could be neutralised with gas-gangrene antitoxin. Further work determined that *C. difficile* could be recovered from patients with PMC and implicated *C. difficile* as the cause of PMC (Bartlett et al, 1978b).

Larson et al (1978), also continued the search for the aetiologic agent and demonstrated that *C. difficile* could be isolated from patients with PMC, but not from control patients. The study showed *C. sordellii* antitoxin to inactivate the toxin in

faecal filtrates and pure culture. Similar studies by George and Symonds (1978), recovered *C. difficile* from patients with PMC and showed that *in vitro* cytopathic effects were neutralised by *C. sordellii* antitoxin. Many studies have since made the association between the use of antibiotics and *C. difficile* colonisation and infection.

1.3. The ecology of *C. difficile*

1.3.1. *C. difficile* in the general environment

C. difficile is thought to be very common in the environment, it has been isolated from numerous sources and as a spore forming organism that is resistant to air, heat and desiccation, it may exist indefinitely in the environment. As the frequency of *C. difficile* in the environment is uncertain, various studies have sought to determine the regularity with which *C. difficile* can be isolated in our general environment. Early reports such as the isolation of *C. difficile* from the gut of a Weddel seal in the Antarctic (McBee, 1960) suggested the presence of *C. difficile* in the environment. However, Riley (1994) failed to isolate *C. difficile* from soil, sand and water samples from rivers and lakes. Kim et al (1981), also failed to isolate *C. difficile* from random soil samples. A large environmental study was carried out around Cardiff, Wales, with the aim of demonstrating how individuals might be exposed to *C. difficile* in everyday life (Al Saif and Brazier, 1996). The study sampled 2580 sites which included water from rivers, lakes, the sea, swimming pools and domestic tap water. Soil samples from parks, play areas, gardens and fields were also subject to investigation. In contrast to the previous studies (Riley et al, 1994; Kim et al, 1981), *C. difficile* was isolated from 36% of water samples and 21.4% soil samples. Al Saif and Brazier (1996), isolated *C. difficile* from tap water as well as lakes, sea-water and

swimming pools. *C. difficile* was also isolated from 2.4% of raw vegetables tested. This comprehensive study by Al Saif and Brazier (1996), used the best culture and enrichment methods available and the study suggests that *C. difficile* is very common in our environment and that we may regularly encounter the organism through normal daily activities.

1.3.2. *C. difficile* in the hospital environment

The presence of *C. difficile* in the hospital environment is well documented and it is likely to have a role in facilitating the spread of the organism. Studies have shown that levels of environmental contamination can vary significantly between wards and within different areas of the same ward. Recently, Fawley and Wilcox (2001), isolated *C. difficile* from 34% and 36% of sites sampled in two general medicine wards for the elderly. Al Saif and Brazier (1996), investigated 380 samples from hospital surfaces and reported 20% to be culture positive for *C. difficile*. Several earlier studies reported isolation rates of 2.1% to 19.6% (Kim et al, 1981), and 7% to 14% from the hospital sites sampled (Fekety et al, 1981). The levels of environmental contamination in hospitals have been shown to be associated with the number of colonised and infected patients in the vicinity of the site sampled (Kim et al, 1981; Fekety et al, 1981; McFarland et al, 1989). Another study investigated the hospital environment of eight patients who were culture positive for *C. difficile,* and control environments where there were no known cases of diarrhoea. *C. difficile* was isolated from 32.5% of sites sampled where patients known to be carrying the organism were situated, and only 1.3% of areas where there were no known cases of diarrhoea (Mulligan et al, 1980).

It is significant to note for comparison that Al Saif and Brazier (1996), isolated *C. difficile* from only 2.6% and 2.2% of surfaces in nursing homes and private residences respectively. These studies indicate a high prevalence of *C. difficile* in the hospital environment which may act as an important reservoir of the organism for cross-infection.

1.3.3. Colonisation and infection of animals with *C. difficile*

Borriello et al (1983), isolated *C. difficile* from 23% of household pets investigated, including dogs, cats, a duck and a goose. Al Saif and Brazier (1996), studied the faeces of farm and domesticated animals, they obtained *C. difficile* from the faeces of a small number (<1%) of farm animals, and from 10.5% of domesticated cats and dogs. A previous study investigated the faeces of animals in two veterinary clinics, *C. difficile* was isolated from 61% in one clinic and 17.5% in the other clinic (Riley et al, 1991). These studies indicate colonisation with *C. difficile* in healthy domesticated animals. However, it has been demonstrated that animals can also suffer from *C. difficile* related diseases, and as with human infection the disease usually results after intestinal disruption with antibiotic therapy. Diarrhoea and fatal colitis linked to lincomycin contaminated grain feed was reported in horses and the likely cause of this disease was thought to be *C. difficile* (Raisbeck, 1991). *C. difficile* has also been reported as the cause of chronic recurrent diarrhoea in dogs (Berry and Levett, 1986), and the association with fatal colits in hamsters after administration with clindamycin is well documented (Bartlett and Gorbach, 1977). Although colonisation and infection with *C. difficile* in animals is common there is no evidence for *C. difficile* as a zoonotic pathogen. The organism is very common in

the environment and it is likely that animals acquire the organism from the environment in a similar way to humans.

These studies indicate that *C. difficile* is an organism which is ubiquitous in the environment and it is encountered by man and other animals on a regular basis. It is therefore hardly surprising that it has found a niche in the hospital environment.

1.4. Epidemiology of *C. difficile*

As discussed, *C. difficile* is common in both general and hospital environments. It is likely that individuals can acquire the organism from numerous environmental sources. The organism is probably carried from time to time by healthy individuals "in transit" as part of their healthy gut flora, and in most cases causes no adverse effects. *C. difficile* is associated with asymptomatic colonisation in healthy adults, individuals in both hospital and the community with a history of antimicrobial therapy and a particularly high number of neonates are asymptomatically colonised with *C. difficile*. Hospitalised adults and babies probably acquire *C. difficile* from the hospital environment.

1.4.1. Asymptomatic colonisation with *C. difficile*

Healthy adults

The isolation rate of *C. difficile* from healthy adults has been shown to vary considerably. The findings from several studies are presented in table 1.1, and show isolation rates to vary from 0-12% in similar patient populations. Nakamura et al (1981), Wilson et al (1982a) and Sharp (1985), reported considerably higher rates of isolation of *C. difficile* than the other studies. The discrepancy of these findings is unclear, Wilson et al (1982a), did not state the source of the normal adults in the

study, and whether or not they had recently received any antimicrobial therapy. A small number of patients investigated by Sharp (1985), had undergone recent exposure to antibiotics. Nakamura et al (1981), investigated a Japanese population and it was thought that the high colonisation rates may reflect ethnic origin and dietary differences. However, Kato et al (2001), recently reported average colonisation rates of 7.6% from several populations of healthy Japanese adults. Kato et al (2001), showed that the colonisation rate could vary between 4.2% and 15.3% in healthy populations of adults indicating that factors other than culture methods and ethnic origin could influence colonisation rates in healthy adults. Despite this, the culture methods, processing and the storage of specimens did vary considerably between the studies presented in table 1.1. For example, Ambrose et al (1985), used a non-specified selective medium and Viscidi et al (1981), used an agar with cefoxitin and cycloserine, the selective agents recommended by George et al (1979). Small variations in selective agents can dramatically affect the sensitivity of the media, and the enrichment of cultures used by Phillips and Rogers (1981), may have significantly enhanced the recovery of the organism. Processing and storage of the faecal specimens may also have affected the isolation rate. Viscidi et al (1981) and Ambrose et al (1985), processed samples and froze them at –40°C before analysis, this perhaps reflects their failure to detect any colonised individuals. When tested for, free cytotoxin was not detected in the faeces of the healthy adult carriers. However, it was shown that *C. difficile* isolates collected from healthy adults had the ability to produce toxin *in vitro* (Aronnson et al, 1985).

Table 1.1. The isolation rates of *C. difficile* the healthy adult population

POPULATION SAMPLED	NUMBER (%) OF INDIVIDUALS POSITIVE FOR *C. DIFFICILE* BY CULTURE	TOXIN(S) IN FAECES	REFERENCE
Healthy adults	0/84 (0)	0/84 (0)	Ambrose et al (1985).
General population	11/594 (1.9)	0/594 (0)	Aronnson et al (1985).
Healthy adults	4/200 (2)	NOT TESTED	Phillips & Rogers (1981).
Normal adults	5/39 (12)	0/39 (0)	Wilson et al (1982a).
Healthy adults	0/60 (0)	0/60 (0)	Viscidi et al (1981).
Healthy adults	49/431 (11.4)	0/431 (0)	Nakamura et al (1981).
Healthy adults	8/72 (11.1)	NOT TESTED	Sharp (1985).
Healthy adults	94/1234 (7.6)	NOT TESTED	Kato et al (2001).

Neonates

The high rate of asymptomatic colonisation of healthy neonates with *C. difficile* is well documented but poorly understood. Many studies have investigated the colonisation rates amongst neonates in hospital nurseries. The results from several studies are shown in table 1.2, and these colonisation rates vary from 10.6%-66.7%. This variation is likely to reflect both culture methodology and genuine variation in the populations investigated. Taffinder et al (1997), investigated the colonisation rates in neonates in a nursery over four different time periods and found that the isolation rate varied from 10% to 57%, indicating that within an individual nursery there can be considerable variation.

The source of neonatal colonisation with *C. difficile* is likely to be environmental. Al-Jumaili et al (1984), isolated *C. difficile* from 71% of infants in a special care baby unit, and related levels of colonisation to the duration of time spent in hospital.

This indicated that the acquisition of *C. difficile* from the environment and from health personnel was a likely possibility. Larson et al (1982), also made an association between environmental contamination and clusters of colonised infants. Other possible sources of *C. difficile* have been implicated. Acquisition from both the gastrointestinal tract and the vagina have been suggested. Hafiz et al (1975), published a study which suggested that a high percentage of women carried *C. difficile* in the vagina. (Larson et al, 1982); Bolton et al (1984), failed to isolate *C. difficile* in such high numbers from the vagina, however they did not use enrichment culture methods. Both Bolton et al (1984) and Larson et al (1982), reported no relationship between vaginal or gastrointestinal colonisation by the mother and neonatal colonisation.

As discussed in section 1.3.2, the hospital environment is often contaminated with *C. difficile* spores. As the environment is thought to be a major reservoir of *C. difficile* for patients who develop AAD in adults, it is likely also to be a reservoir for the colonisation of neonates.

A number of factors have been suggested to be influential in the colonisation of neonates. Infants lack the complex flora of older children and adults; this may explain how the organism can proliferate in the neonatal and infant gut. The incidence of colonisation with *C. difficile* in children has been shown to decrease with age. Viscidi et al (1981), showed that colonisation with *C. difficile* dropped from 29% of neonates to 9% of infants aged between 3-24 months old. Work carried out by Cooperstock et al (1983), suggested that breast fed babies were less likely to be colonised with *C. difficile* than formula fed babies, suggesting that the infant is protected by maternal antibody in the breast milk. However, Donta and Myers

(1982), showed that colonisation with *C. difficile* was more frequent in breast fed babies.

Table 1.2. Isolation rates of *C. difficile* from neonates

NUMBER (%) OF INDIVIDUALS POSITIVE FOR		
C. DIFFICILE BY CULTURE	TOXIN(S) IN FAECES	REFERENCE
46/150 (30.7)	22/150 (14.7)	Bolton et al (1984).
8/75 (10.6)	NOT TESTED	Brettle and Wallace (1984).
76/114 (66.7)	NOT TESTED	Delmée et al (1988).
NOT TESTED	11/105 (10.5)	Donta and Myers (1982).
4/10 (40)	NOT TESTED	Hall and O'Toole (1935).
32/61 (52.5)	NOT TESTED	Larson et al (1982).
28/182 (15.4)	NOT TESTED	Snyder (1940).
31/50 (62)	NOT TESTED	Tabaqchali et al (1984a).
28/99 (28)	NOT TESTED	Taffinder et al (1997).
6/22 (27.3)	1/22 (4.5)	Torres et al (1984).
13/45 (28.9)	12/45 (26.7)	Viscidi et al (1981).

As the summary of studies in table 1.2 shows, *C. difficile* toxins were detected in a large number of asymptomatically colonised infants. The presence of toxin in the adult gut usually indicates infection and is rarely detected in the faeces of asymptomatic colonised adults. However, toxin is often detected in the faeces of asymptomatic infants, the reason for this remains to be determined. Several suggestions for the presence of toxin in the faeces of asymptomatically colonised infants are lack of receptors for toxin or masked receptors in the infant gut. However,

this does not explain why the organism produces toxin in detectable quantities in the infant gut.

Adults with a history of antimicrobial therapy

Studies have shown an increase in the rate of asymptomatic colonisation with *C. difficile* in patients with a history of antibiotic use. Viscidi et al (1981), showed that 21% of patients carried *C. difficile* after antibiotic therapy. Another study demonstrated the acquisition of *C. difficile* during or after treatment with cefoxitin by 5 of 6 patients (Mulligan et al, 1984). It was shown that 15 of 78 volunteers acquired *C. difficile* after only one parenteral dose of cephalosporin (Ambrose et al, 1985). Bartlett (1992), reported that 10-25% of patients receiving antibiotics were colonised with *C. difficile*.

Individuals with cystic fibrosis are colonised with *C. difficile* at a higher rate than the general population, this is likely to reflect the increased exposure to antibiotics in these groups of individuals. Cystic fibrosis patients are almost always receiving an antibiotic regime for prophylactic purposes, however, symptoms of AAD are rarely reported in cystic fibrosis patients (Wu et al, 1983). In a study of cystic fibrosis patients receiving antibiotics, 50% were shown to carry *C. difficile*, compared to none of the cystic fibrosis patients not receiving antibiotics. (Wu et al, 1983). In a study by Peach et al (1986), 32% of cystic fibrosis patients carried *C. difficile* asymptomatically compared to 17% of control patients on antibiotics. The relatively high colonisation rate of *C. difficile* in cystic fibrosis patients is likely to be a result of continual antibiotic therapy and is therefore not surprising.

1.4.2. Infection with *C. difficile*

Hospital-acquired CDAD

AAD and PMC related to *C. difficile* is a significant hospital-acquired infection that predominately affects elderly patients who have undergone antibiotic therapy. The rates of infection vary from hospital to hospital and between wards within hospitals. Rates of confirmed *C. difficile* infection varied between 0.76 and 6% in a report that investigated nine different health authorities in the UK (Djuretic et al, 1999). Karlstrom et al (1998), reported an overall rate of *C. difficile* infection at 17 in 10,000 patients in a survey of 21 Swedish hospitals. The study also demonstrated the varying incidence of infection in different types of ward. The highest rate at 95 in 10,000 cases occurred in geriatric and rehabilitation wards, 16 in 10,000 cases occurred in oncology wards and the lowest rates, 2 in 10,000 occurred in ear/nose/throat, dermatology, gynaecology and neurology combined.

Any hospitalised patient undergoing antibiotic therapy is at risk of developing CDAD, however it is generally accepted that elderly patients are most at risk of acquiring the infection. Other immunocompromised and debilitated groups including cancer patients undergoing chemotherapy and steroid treatment, and Human Immunodeficiency Virus (HIV) positive patients are also at increased risk. A number of risk factors relating to age, underlying illness and medications, including the use of specific antimicrobial agents are thought to influence the development of disease. The importance of these factors will be discussed in detail in section 1.5.

C. difficile associated illness has become an increasing problem since the 1980s. The Public Health Laboratory Service for England and Wales reported a steep increase in the number of laboratory identifications of *C. difficile* over the last decade. The

number of laboratory identifications of *C. difficile* in England and Wales has risen from 682 in 1998 to 4375 in 2000; this increase is illustrated in figure 1.1 (www.phls.co.uk). Similar increases have also been shown in Scotland (data obtained from The Scottish Centre for Infection and Environmental Health); this is illustrated in figure 1.2. It should be noted that *C. difficile* associated disease is a reportable infection in Scotland, but not in England and Wales.

Figure 1.1. Laboratory reports of *C. difficile* in England and Wales 1988-2000

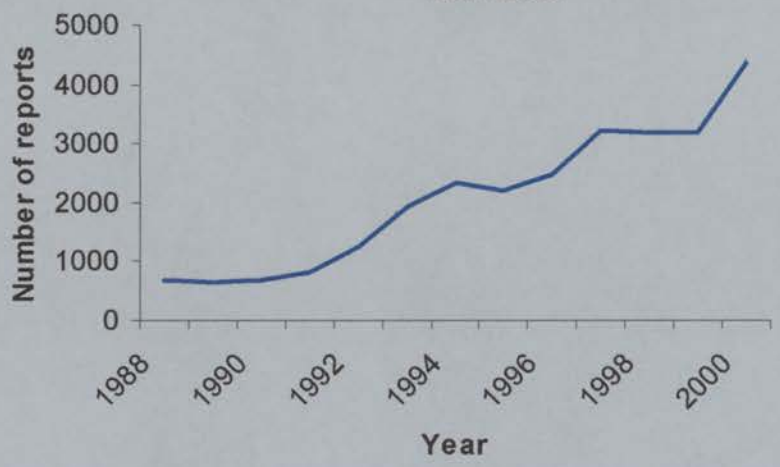
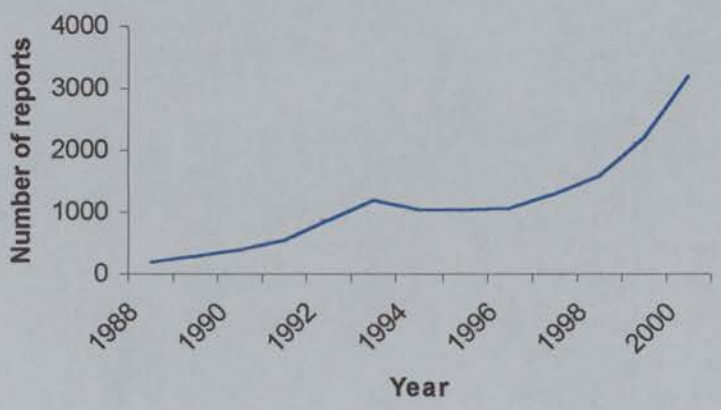


Figure 1.2. Laboratory reports of *C. difficile* in Scotland 1988-2000



organism and its virulence factors. It is the events of these interactions that determine the pathology and outcome of the disease.

Infection with *C. difficile* requires the initial disruption of the gut flora, usually with antibiotics, followed by exposure to *C. difficile*. The bacterium, *C. difficile* colonises the gut and expresses a number of virulence factors which may contribute to the pathology of disease. *C. difficile* toxins A and B are the major virulence factors and they are responsible for the principal pathological effects of *C. difficile* disease. Other putative virulence factors such as capsules, flagella, hydrolytic enzymes, other toxic factors and the S-layer proteins (SLPs) may be involved in the colonisation process, and the virulence associated with *C. difficile*.

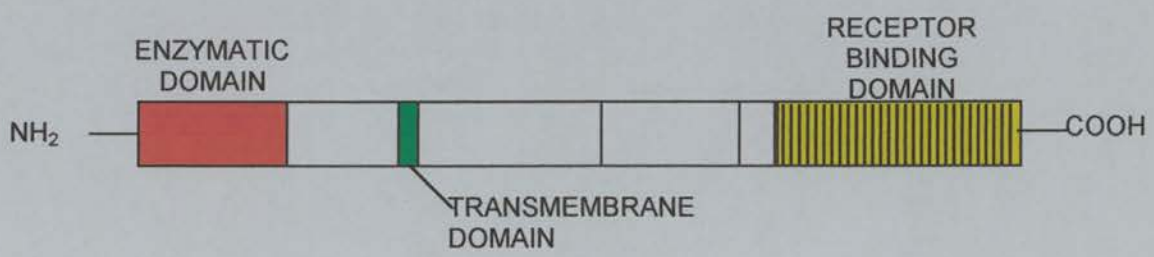
1.7.1. The *C. difficile* toxins- toxin A and toxin B

The major pathology of CDAD is a result of the actions the two potent cytotoxins, *C. difficile* toxins A and B. These toxins belong to the family of Large Clostridial cytoToxins (LCTs).

The structure of toxin A and toxin B

Both toxins are large, single-chained peptides, toxin A has a molecular mass of 308 kilo Daltons (kDa), and toxin B has a mass of 250 kDa. The toxins have an average of 49% amino acid sequence homology (von Eichel-Streiber, 1995), and each toxin has three functional domains, as shown in figure 1.3.

Figure 1.3. The general structure of the toxin A and toxin B peptide
Adapted from Moncreiff and Wilkins (2001).



were frequently associated with community acquired cases of CDAD.

Cephalosporins are frequently used to treat community-acquired pneumonia, and as the elderly often suffer from such illnesses they are likely to be at an increased risk of acquiring community acquired *C. difficile*, and should be closely monitored by their GP.

1.5. Risk factors associated with CDAD

A large number of potential risk factors for CDAD relating to medication, medical diagnoses, medical procedures, underlying diseases and host factors have been suggested. Epidemiological investigations of possible risk have produced conflicting results. However approaches to studies have varied, different statistical methods have been used and many early reports did not account for confounding factors.

The multifactorial nature of CDAD makes analysis for influential risk factors more difficult as many factors and many combinations of factors may induce patient susceptibility, it is important that analysis accounts for any confounding interactions of risk factors. Accurate identification of the most significant and most frequently associated risk factors would be beneficial in the development of control and treatment strategies.

Age and antibiotic use are well-established risk factors for *C. difficile* colonisation and disease. The significance of other factors such as medications other than antibiotics, diagnostic procedures, underlying diseases and immune status remain undetermined.

1.5.1. Age

The association of *C. difficile* infection with increasing age is well documented. A number of epidemiological studies have determined age as a significant risk factor for the disease (McFarland et al, 1990; Buchner & Sonnenberg, 2001). Aronnson et al (1985), reported that the incidence of CDAD was significantly higher in patients over 60 years of age.

Elderly patients are also more likely to have been exposed to the hospital environment, and other possible risk factors, as a result of underlying diseases and poor general health. Their poor health may affect the efficiency of their immune system and their ability to deal with infection.

1.5.2. Antibiotic use

Most antibiotic agents have been associated with the CDAD. The disease was initially associated with clindamycin use, however broad-spectrum agents such as cephalosporins and ampicillin most frequently provoke the disease (Bartlett, 1981; Aronnson et al, 1985)

The normal gut flora offers protection from colonisation by pathogenic bacteria. This protective effect is referred to as "colonisation resistance." The effect of the gut flora against *C. difficile* growth and toxin production has been shown *in vitro* (Borriello and Barclay, 1986). Treatment with antibiotics diminishes the gut flora and therefore decreases the effect of colonisation resistance, this exposes a patient to colonisation by pathogenic bacteria including *C. difficile*.

Cephalosporins have been implicated as the cause of CDAD in many studies, and evidence suggests that they are potent inducers of *C. difficile* colonisation and disease. Aronnson et al (1985), found that cephalosporins were implicated 40 times

more often than narrow spectrum penicillins, and Silva et al (1984), reported that cephalosporin use was implicated in 71 of 130 cases of CDAD. Impallonemi et al (1995), reported a sudden increase in the number of cases of CDAD in geriatric patients following a 20-fold increase in the use of cefotaxime, the number of cases dropped when cefotaxime use was terminated. Several studies have indicated that antibiotic policies restricting the use of cephalosporins can significantly reduce the incidence of infection. Cartmill et al (1994), reduced the use of cephalosporins during an outbreak, the number of cases of CDAD were reduced and a decreased rate of disease was maintained. McNulty et al (1997), showed that controlled use of cefuroxime in an elderly care unit reduced and maintained a low level of CDAD. Ampicillin and amoxycillin have long been associated with CDAD (Freeman and Wilcox, 1999). Silva et al (1984), showed ampicillin use in 38 of 130 cases of CDAD, and Bartlett (1981), demonstrated that 109 of 329 patients with *C. difficile* disease had been exposed to ampicillin.

Other antimicrobial agents including erythromycin, tetracycline, other penicillins (Bartlett, 1981), clarythromycin (Braeggar and Nadal, 1994) and co-trimoxazole (Gordin et al, 1994) have been implicated. Almost all antibiotics have been implicated at some time, and due to the multiple antibiotic regimes it can be difficult to determine the inciting agent. The association with the 4-fluoroquinolones is rare despite their wide-spread use following their introduction in the 1980s (Freeman and Wilcox, 1999). Golledge et al (1992), failed to isolate *C. difficile* from 117 patients who had received ciprofloxacin treatment. Studies have shown that ciprofloxacin is non-bactericidal in anaerobic conditions, and may therefore not affect the protective anaerobic components of the gut flora (Smith et al, 1988).

However, it is the cephalosporins, clindamycin, ampicillin and amoxycillin which most frequently induce disease.

CDAD is usually associated with antibiotic treatments, however the use of single dose surgical prophylaxis has also been implicated. Privitera et al (1991), showed that of 108 volunteers undergoing elective surgery, 23% of those who received a single dose of cephalosporin acquired *C. difficile*, however only 3.3% of those who received mezlocillin acquired *C. difficile*, none of the patients developed diarrhoea. Ambrose et al (1985), also showed a higher acquisition rate of *C. difficile* in patients who received a single dose of a cephalosporin compared to those who received a single dose of penicillin.

Antibiotics are clearly a predisposing factor for the development of CDAD. In addition to the diminishing effect on the gut flora allowing colonisation, the antibiotics may also induce stress responses which can upregulate toxin production, increase adherence and expression of virulence factors (Freeman and Wilcox, 1999).

1.5.3. Medical procedures and medication

Medical procedures

A number of medical procedures which may allow entry of *C. difficile* to the intestinal tract and/or reduce the "colonisation resistance" in the gut allowing *C. difficile* to colonise and proliferate have been suggested to increase the risk of developing *C. difficile* infection.

Nasogastric feeding tubes have been associated with *C. difficile* infection, Simor et al (1993), investigated patients in a long term care facility and logistic regression modelling showed nasogastric feeding to be significant in those patients with CDAD when compared to those without. Pierce et al (1982), investigated ten cases of *C.*

difficile, three patients had received nasogastric feeding for two or more days, and it was suggested that this may be an influencing factor. However, MacFarland et al (1990), investigated patients in a general medicine ward over an 11-month period and found that the use of nasogastric feeding tubes was not significant in colonised patients or patients with CDAD.

One study reported a significant reduction in the number of cases of *C. difficile* infection following the replacement of reusable rectal thermometers with single use disposable thermometers (Brooks et al, 1992). This indicates that *C. difficile* may be allowed to enter the gastrointestinal tract via contaminated instruments, thus sigmoidoscopy and endoscopy procedures may carry a risk for *C. difficile* infection. Both McFarland et al (1990) and Pierce et al (1982), reported that the use of enemas was significant in patients with CDAD. Pierce et al (1982), also reported that gastrointestinal surgery could predispose patients to *C. difficile* disease. However, Gerding et al (1986), did not find gastrointestinal surgery to be significantly associated with *C. difficile* infection, and in a one year prospective study of 241 patients who had undergone gastrointestinal surgery only nine developed CDAD. A number of case reports have linked gastrointestinal surgery with CDAD, however other factors such as antibiotic use were not taken into account.

Medication (other than antibiotics)

Antacids and anti-ulcer H_2 antagonists have been implicated in *C. difficile* disease. It is thought that as these medications increase the pH in the stomach that this could facilitate the entry of *C. difficile* to the gastrointestinal tract. McFarland et al (1990), found that the use of gastric acid suppressants was a significant factor in the colonisation of patients, but was not significant in those patients with CDAD.

Simor et al (1993), investigated patients in a long term care facility and found that the use of anti-ulcer drugs did not influence infection in these patients. Shah et al (1998), investigated the records of patients who had been investigated for *C. difficile*. The study revealed that usage of gastric acid suppressants was similar in patients who tested positive and patients who tested negative for *C. difficile*. It is likely that the low pH of the gastric secretions have no effect on *C. difficile* spores. MacFarland et al (1990), reported that the use of stool softeners was significant for colonisation and CDAD, however laxatives were not. Simor et al (1993), also reported that laxatives were not significant in CDAD. Cancer chemotherapy drugs and steroids have also been associated with CDAD.

1.5.4. Underlying diseases and immune status

Gastrointestinal disease

Harbarth et al (2001), studied patients in a cardiac surgery ward who developed *C. difficile* and found that gastrointestinal disease was an associated risk factor. Greenfield et al (1983), reported that *C. difficile* could be isolated in significantly more patients with inflammatory bowel diseases than in healthy individuals with normal bowel habits. Patients with inflammatory bowel diseases are more frequently exposed to antibiotics and this may reflect the high isolation rate from these patients. Keighley et al (1982), reported that patients with inflammatory bowel disease did not develop CDAD unless they were exposed to antibiotics and Gerding et al (1986), also found no association with colonic diseases and the development of CDAD.

Cancer

Milligan and Kelly (1979), reported fatal PMC in five patients with leukaemia undergoing chemotherapy and antibiotic treatment, and suggested strict control of antibiotic regime in patients with serious underlying illness. Several later reports linked the possible suppression of the gut flora by anti-cancer chemotherapy and histopathological changes in the gut mucosa with PMC (Fainstein et al 1981; Cudmore et al 1982). A number of studies have since made the association with cancer chemotherapy and CDAD.

As well as the effects of anti-cancer chemotherapy, cancer patients often have reduced immune status as a result of steroid therapy. Steroids have been implicated in CDAD, as these drugs are often combined with chemotherapy and can diminish the effects of the immune system.

The importance of immune status is reflected in the relatively high incidence of *C. difficile* infection reported in immunocompromised patients including HIV positive and acquired immune deficiency syndrome (AIDS) patients. Hutin et al (1993), reported CDAD in 4.1% of HIV infected patients, the incidence of *C. difficile* diarrhoea in HIV infected patients was reported by Tacconelli et al (1999), to be 3.1%, almost double that of non-HIV infected patients in a large university hospital. However, the high frequency of hospitalisation, cancer therapy and antibiotic use in HIV patients are also important influential factors.

Immune status

A reduced immune status is a common factor in the groups of patients who most frequently suffer from *C. difficile* infection. Elderly and debilitated patients often have impaired immune function, the immune systems of elderly patients become less

efficient and various functions of the immune system become defective with the progression of age (Haeney, 1994). Elderly, HIV-positive and cancer patients with underlying health problems are more likely to be hospitalised and are more frequently exposed to a variety of the potential risk factors.

However, as discussed, one of the first lines of defence against any gastrointestinal pathogen is "colonisation resistance." It is the reduction in colonisation resistance by antibiotics and other drugs that allow the colonisation of the gut with *C. difficile*. Barclay and Borriello (1986), showed that the colonisation resistance in elderly patients was diminished suggesting that they require less compromise before developing the disease.

Following colonisation with *C. difficile* the role of the immune system in eliminating the infection is undetermined. Several investigators have studied antibody responses to *C. difficile* toxins (Johnson et al, 1992; Warny et al, 1994) and to cell surface proteins (Pantosti et al, 1989). Johnson et al (1992), showed that immunoglobulin G (IgG) and immunoglobulin A (IgA) responses to *C. difficile* toxin A vary greatly between patients and controls. Warny et al (1994), showed that IgG and IgA levels to toxin A were higher in those patients that suffered only from a single episode of diarrhoea compared to those who suffered from relapses. Kyne et al (2001), also showed higher antibody levels to toxin in patients who did not suffer from relapses.

Many factors have been shown to significantly influence the development of CDAD in specific populations. Factors such as the level of environmental contamination and proximity to infected patients are thought to be important in the spread of infection, however this is difficult to investigate. Evidence would also suggest that host factors, including age, play a major role in determining patient susceptibility. Host factors

cannot be controlled, however, by the identification of the influential host factors and other risk factors effective control strategies may be developed.

Starr et al (1997), proposed a "herd immunity" model for *C. difficile* infection. The model suggests that within a ward ("herd") there is an overall state of resistance to *C. difficile*. However, if the number of resistant patients in the ward drops below a critical level then cases of *C. difficile* will occur. By identifying the factors which shift the equilibrium of resistant patients below the critical level, and careful attention to case-mix to account for uncontrollable host factors, effective control of *C. difficile* infection may be achieved.

1.6. The spectrum and clinical presentations of *C. difficile* disease

The clinical manifestations associated with *C. difficile* colonisation range from asymptomatic colonisation (discussed in section 1.4), mild diarrhoea and non-pseudomembranous colitis to fulminant colitis which can result in death. Most patients suffer only from the mildest forms of the disease and fortunately the more serious outcomes are relatively rare (Kelly and LaMont, 1991). The reasons for the variation in the severity of *C. difficile* infection are unclear, however host factors are likely to be of paramount importance.

Antibiotic-associated diarrhoea (AAD)

It is common for patients using antibiotics to develop mild symptoms of diarrhoea due to the disturbance of the normal bacterial flora. The normal fermentation process in the gut is altered during antibiotic treatment resulting in unabsorbed carbohydrate in the gut. This carbohydrate in the gut binds water and causes diarrhoea which is relieved on the termination of antibiotic therapy and the return of the normal gut

flora (Rao et al, 1988). AAD can also be a result of colonisation with *C. difficile*. The diarrhoea is usually mild to moderate and can be accompanied by abdominal cramping, and usually ceases upon termination of the offending antibiotic therapy requiring no further treatment (Kelly et al, 1994a).

C. difficile colitis (without the formation of pseudomembranes)

C. difficile colitis without the production of pseudomembranes is more serious than the mildest form of AAD. Patients suffer from abdominal pain, cramping and much more profuse and debilitating diarrhoea. Other symptoms may also include malaise, anorexia, nausea, dehydration and fever. Colonic bleeding is rare and sigmoidoscopy reveals patchy colitis (Kelly et al, 1994a).

Pseudomembranous Colitis (PMC)

Symptoms of PMC are similar to, but more severe than the symptoms associated with the milder form of colitis. Sigmoidoscopy reveals the yellow plaques associated with PMC on the colon and rectum mucosa. In severe cases the pseudomembrane may cover large areas of the colorectal mucosa (Kelly et al, 1994a).

Fulminant colitis

This only occurs in about three percent of patients but can result in gut perforation and megacolon requiring the need for surgical intervention, in the most severe cases death may occur. Patients suffer from abdominal pain, cramping, severe diarrhoea and gut distension, high temperatures and leukocytosis (Kelly et al, 1998).

1.7. The virulence factors associated with *C. difficile*.

Any infectious disease is a complex set of interactions between host and pathogen. The host immune system triggers a series of events in response to the infecting

organism and its virulence factors. It is the events of these interactions that determine the pathology and outcome of the disease.

Infection with *C. difficile* requires the initial disruption of the gut flora, usually with antibiotics, followed by exposure to *C. difficile*. The bacterium, *C. difficile* colonises the gut and expresses a number of virulence factors which may contribute to the pathology of disease. *C. difficile* toxins A and B are the major virulence factors and they are responsible for the principal pathological effects of *C. difficile* disease. Other putative virulence factors such as capsules, flagella, hydrolytic enzymes, other toxic factors and the S-layer proteins (SLPs) may be involved in the colonisation process, and the virulence associated with *C. difficile*.

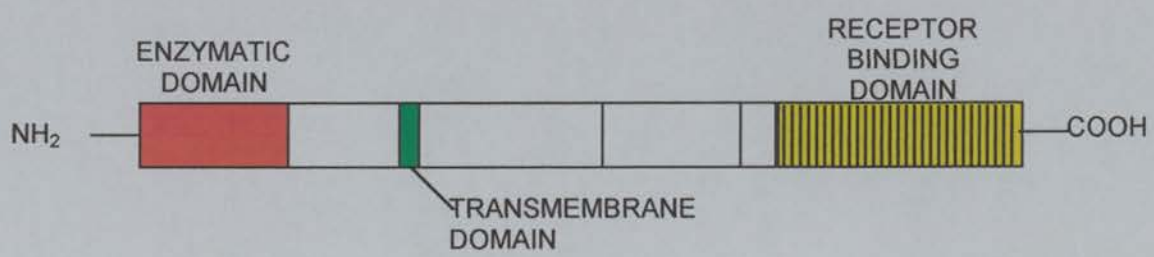
1.7.1. The *C. difficile* toxins- toxin A and toxin B

The major pathology of CDAD is a result of the actions the two potent cytotoxins, *C. difficile* toxins A and B. These toxins belong to the family of Large Clostridial cytoToxins (LCTs).

The structure of toxin A and toxin B

Both toxins are large, single-chained peptides, toxin A has a molecular mass of 308 kilo Daltons (kDa), and toxin B has a mass of 250 kDa. The toxins have an average of 49% amino acid sequence homology (von Eichel-Streiber, 1995), and each toxin has three functional domains, as shown in figure 1.3.

Figure 1.3. The general structure of the toxin A and toxin B peptide
Adapted from Moncreiff and Wilkins (2001).



The three functional domains of *C. difficile* toxins A and B:

(i) The enzymatic domain: This is located at the N-terminus and catalyses the glucosylation of guanosine 5'-triphosphate (GTP)-binding proteins (Hofmann et al, 1997).

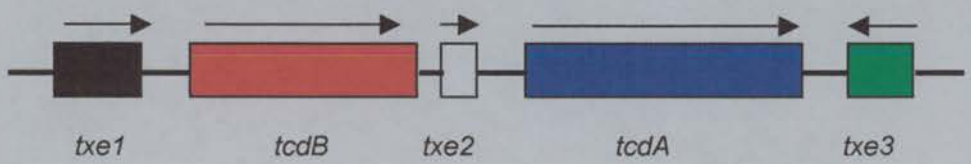
(ii) The transmembrane domain: Centrally located, this domain is likely to mediate the translocation of the toxin to the cytosol, however this has not been proven (Just et al, 2000).

(iii) The receptor binding domain: Located at the C-terminus, this domain is constructed of repeat motifs and mediates binding to specific carbohydrate structures (Pothoulakis et al, 1991; Pothoulakis et al, 1996; Krivan et al, 1986; Clark et al, 1987).

The pathogenicity locus (PaLoc)

Toxins A and B are coded on a 19.6 kb pathogenicity locus (PaLoc). The PaLoc encompasses the two toxin genes (*tcd A* and *tcdB*) and three accessory genes, *txe1, txe2 and txe3*, this is shown in figure 1.4.

Figure 1.4. The pathogenicity locus of *C. difficile*
Adapted from Moncreiff and Wilkins (2000).



(Arrows indicate the direction of transcription)

The possible functions of the accessory genes:

txe1 and *txe2* both reach peak transcription in stationary phase, *txe1* may positively regulate the toxin gene promoters.

txe 2 is structurally and functionally similar to holin proteins which may facilitate the release of toxin A and B, via cell lysis (Soo Tan et al, 2001).

txe3 – transcript levels peak in exponential phase, it may be a negative regulator (Moncrief and Wilkins, 2000).

A number of toxin variant strains have been identified (Lyerly et al, 1992, Borriello et al, 1992a; Deptire et al, 1993). These toxin variant strains possess the PaLoc. However, it is different when compared to the pathogenicity locus from the reference strain (VPI 10463). These differences result from deletions in the genes and the outcome can be the production of toxin B only (tox A-B+), or failure to produce either toxin (tox A-B-). However, some variant strains can still produce both toxins A and B (Rupnik et al, 2001).

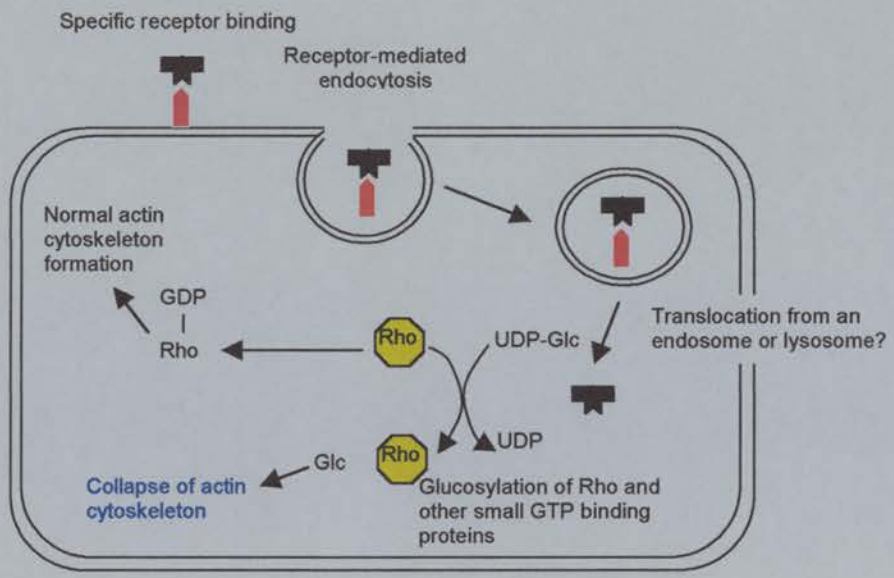
Cellular intoxication with toxin A and toxin B

As previously mentioned both toxin A and toxin B are potent cytotoxic GTP-ase glucosylating proteins. The cytotoxic activity of toxin A and toxin B results from the glucosylation of small GTP-binding proteins such as Rho, Ras and Cdc42, which leads to disruption of the cellular cytoskeleton (Just et al, 1995 a & b).

The toxins bind to the cell surface via a specific receptor, Toxin A has been shown to bind to carbohydrate domains on rabbit sucrase-isomaltase, rabbit erythrocytes and to specific carbohydrate domains on human red blood cells (Pothoulakis et al, 1991; Krivan et al, 1986; Clark et al, 1987). However, the specific carbohydrate receptor

for toxin A on human colonic cells has yet to be determined, and the receptor for toxin B is not known. The toxin enters the cells by receptor mediated endocytosis (von Eichel Streiber et al, 1991). Once in the cytosol the toxins glucosylate small GTP-binding proteins such as Rho and Rac (Just et al, 1995 a & b). The small GTPases are modified and inactivated, thus affecting the signalling pathways within the cell and ultimately leading to the collapse of the actin cytoskeleton. This sequence of events is summarised in Figure 1.5.

Figure 1.5. Cellular intoxication by *C. difficile* toxins A and B
Adapted from Thelestam and Chaves-Olarte (2000).



Action of toxins A and B on the intestine

Toxin A exhibits both enterotoxic and cytotoxic activity, and toxin B has cytotoxic activity 1000 times more potent than toxin A (Lyerly and Wilkins, 1995). Toxin B alone is not active in rabbit intestinal loops assays, however when injected with sub-

lethal amounts of toxin A, death occurs. This indicates that the two toxins act synergistically (Lyerly and Wilkins, 1985).

The *C. difficile* toxins induce a strong inflammatory reaction and it has been shown that both toxins directly and indirectly affect immune cells. It is thought that the binding of toxin A and toxin B to the receptors on the intestinal epithelial cells elicits a signal, which activates fluid secretion and inflammation by both the activation of the enteric neuronal system and the immune response.

Activation of the immune response

Toxin mediated monocyte and macrophage infiltration

Toxin A has been shown to elicit a dose dependent stimulation of neutrophil migration and recruitment (Kelly et al, 1994b). It has been shown that cultured monocytes release tumour necrosis factor-α (TNF- α) upon stimulation with toxins A and B, and that toxin B is 1000 times more potent than toxin A in the same system (Souza et al, 1997). Human monocytes produce IL-8, a neutrophil chemotactic factor upon exposure to *C. difficile* toxins A and B (Linevesky et al, 1997). Mast cells have also been shown to play a role in the neutrophil recruitment. Calderon et al (1998), showed that mast cells produce TNF- α upon stimulation with toxin A, but that prolonged exposure of the mast cells to toxin A impaired their function, affecting the release of inflammatory mediators. This could potentially diminish the effects of the immune response and facilitate disease progression.

Neuronal activation

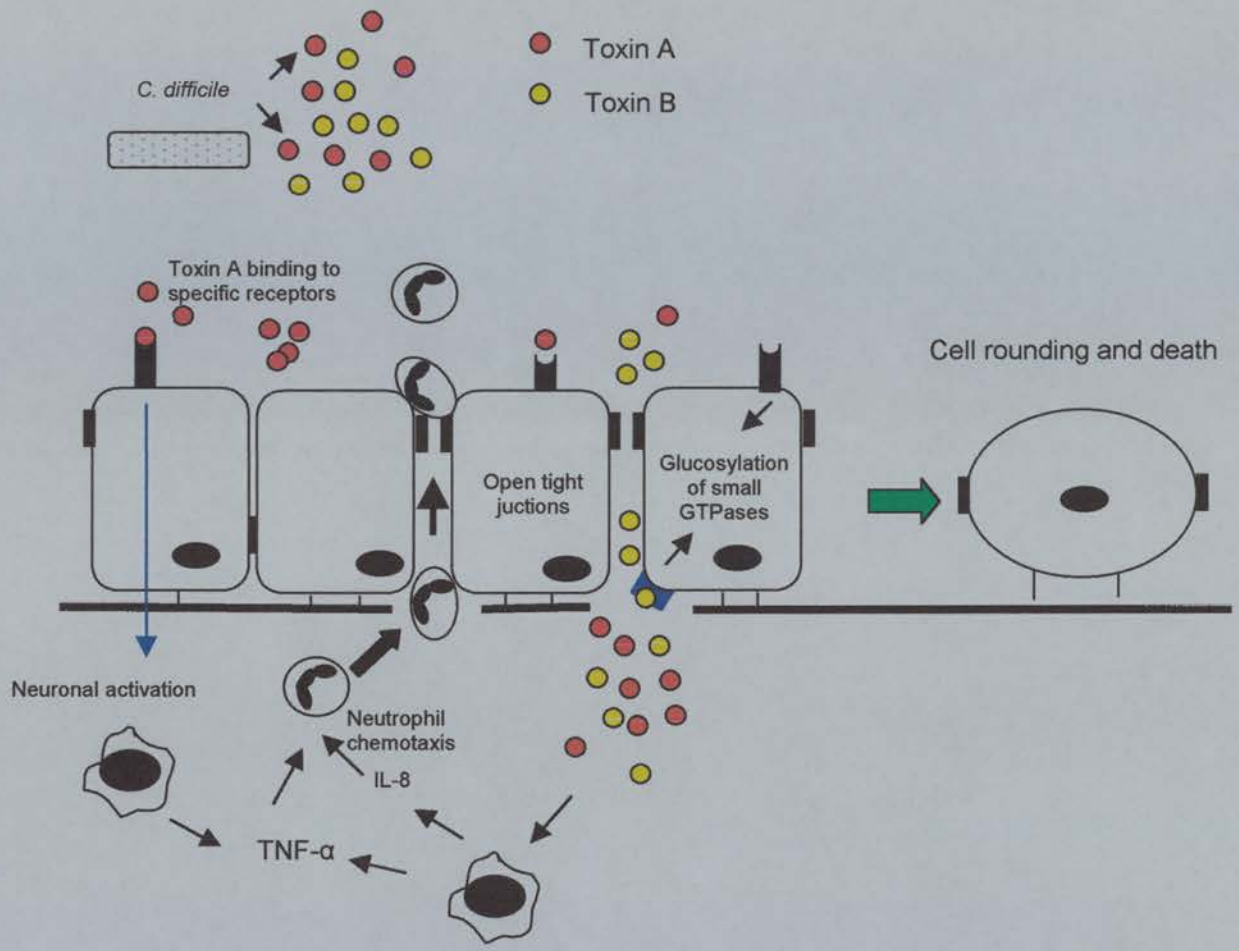
An early study by Castagliuolo et al (1994), indicated that the neuronal system may be involved in the inflammation resulting from *C. difficile* toxins. Pothoulakis et al (1994), demonstrated that inflammation decreased when rats were pre-treated with a

substance P (SP) antagonist before ileal loops were exposed to toxin A. SP is a major neurotransmitter, the activation of which results in secretion, inflammation and tissue damage. Castagliuolo et al (1997), subsequently showed that in rats, toxin A caused an increase in SP response in mucosal scrapings and lumbar dorsal root ganglia. Recently, Keates et al (1998), showed that another neurotransmitter, calcitonin gene-related peptide (CGRP) was involved in the inflammation response induced by toxin A. Castagliuolo et al (1998), showed that intestinal epithelial cells release macrophage inflammatory protein (MIP-2) after exposure to toxin A. MIP-2 then activated sensory nerves to release SP and CGRP which stimulates neutrophils. These studies provide good evidence for the neuronal involvement in the inflammation and fluid secretion induced by process of *C. difficile* toxins.

The pathology of *C. difficile*

The actions of the *C. difficile* toxins are summarised in figure 1.6. The current understanding of the pathogenesis of *C. difficile* resulting from the action of the toxins indicates that following intestinal colonisation with *C. difficile*, toxin A binds to specific carbohydrate receptors on the intestinal epithelium. As a result of the toxin effects on the cellular cytoskeleton, the tight junctions open. This process allows toxin B to pass through the tight junctions where it is taken up by enterocytes, and as well as causing the death of cells, the toxins upregulate the recruitment of neutrophils by both the upregulation of neutrophil chemotactic factors and neuronal activation. The neutrophils can pass through the tight junctions to the gut mucosa where they release inflammatory mediators resulting in mucosal inflammation.

Figure 1.6. Action of the *C. difficile* toxins on the intestinal epithelium
Adapted from Thelestam and Chaves-Olartes (2000).



As cells round and are shed into the lumen of the gut, an ulcer forms on the mucosal surface, and mucus and inflammatory cells flow out through the ulcer forming the pseudomembrane. The pseudomembrane contains cellular debris, fibrin and mucin. They appear on the colonic or rectal mucosa as yellow-white plaques of 0.2-2cm in diameter. These plaques are usually interspersed with normal tissue, however in severe cases a confluent pseudomembrane can cover the entire mucosal surface of the affected area. Usually it is only the epithelium and lamina propria which are

affected, although deeper tissue damage can occur in severe cases (Price and Davies, 1977)

The normal patchy distribution of the pseudomembrane can be described by differing local concentrations of toxin. Reigler et al (1995), showed that mucosal strips exposed to toxin *in vitro* under low concentrations of toxin produced patchy lesions, and as the concentration of toxin increased that patches became almost confluent.

1.7.2. Capsules

Polysaccharide capsules are well-defined bacterial virulence determinants. They can offer protection against immune recognition and phagocytosis by preventing opsonisation, by antigenic variation and by mimicking host molecules.

Davies and Borriello (1990), were the first to demonstrate the production of a capsule by *C. difficile in vitro*. They showed all 15 strains of *C. difficile* tested to produce capsular material. Another investigation has shown *C. difficile* to be very resistant to phagocytosis. Both toxigenic and non-toxigenic strains were resistant to phagocytosis and the removal of cell-surface carbohydrates had no effect on phagocytosis. It was therefore suggested that the capsule might act as an antiphagocytic factor (Dailey et al, 1987), thus contributing to the virulence of *C. difficile*. However, phagocytosis is unlikely to play an important role in the gut mucosa.

1.7.3. Hydrolytic enzymes

The presence of hyaluronidase, chrondroitin sulphatase, gelatinase and collagenase was detected a clinical isolate of *C. difficile* (Steffen, 1981). Popoff and Dodin (1985), investigated 25 *C. difficile* strains for neuraminidase production and failed to detect it in any of the strains tested. Seddon et al (1990), investigated hyaluronidase,

chondroitin-4-sulphatase, heparinase, collagenase and protease production from 30 strains of *C. difficile* with different degrees of virulence in the hamster model. Not all enzymes were detected in all strains, but the study showed that some strains of *C. difficile* produced some of these hydrolytic enzymes and an association between enzyme production and virulence was evident (Seddon et al, 1990). It is likely that these hydrolytic enzymes contribute to tissue damage, thus releasing nutrients and potentially revealing receptors which could facilitate adhesion and/or toxin binding.

1.7.4. Flagella

Flagella are often associated with motility and, together with chemotaxis, can increase the efficiency with which bacteria can contact the host mucosal surface and penetrate the mucous layer. Little research into the role of the flagella of *C. difficile* has been carried out. However, Tasteyre et al (2001a), showed that non-flagellated strains of *C. difficile* associate with mouse caecum to an extent ten-fold less than flagellated strains. The study also showed that the two flagella proteins adhered *in vitro* to mouse caecal mucus, thus suggesting a role in adherence. It was also shown that the FliD (the flagellar cap protein) is very well conserved between many different serotypes of *C. difficile*, suggesting that it has a very specific function Tasteyre et al (2001b).

1.7.5. Other toxic factors

C. difficile has also been shown to produce toxic factors other than toxins A and B. Justus et al (1982), described a toxic substance distinct from *C. difficile* toxins A and B which altered motility and caused changes in the electrical potential of the ligated rabbit intestine.

C. difficile also produces an ADP-ribosyltransferase (binary toxin) which acts in the same way as *C. botulinum* C2 toxin and *C. perfringens* E iota toxin (Popoff et al, 1988). Perelle et al (1997), identified the two genes which transcribed the two components of the toxin, the sequence of which was highly homologous to *C. perfringens* iota toxin. Stubbs et al (2000), investigated the production of the binary toxin in 170 *C. difficile* strains and determined that only toxin variant strains possessed the binary toxin genes. The role of the binary toxin is unclear. It is possible that this toxin can enhance the virulence potential of the organism. The predominant ribotypes isolated from cases in the UK do not possess the binary toxin, although strains producing this toxin have been isolated from patients in the UK and the US.

1.7.6. S-layer proteins

C. difficile possesses two S-layer proteins (SLPs) of different molecular mass (Kawata et al, 1984). The S-layer is a common feature on many Gram-positive and Gram negative bacteria and is composed of protein or glycoprotein sub-units which make up a regularly ordered crystalline array (Sleytr and Messner, 1988). All S-layers share general features; the proteins are relatively large, have a crystalline structure and they self-assemble. There is however a lot of variation in the lattice formats and the molecular weight of the proteins forming these S-layers between species as well as within species (Sleytr and Messner, 1988).

It is presumed that because the SLPs are one of the most abundant bacterial proteins that they must have a role, otherwise the energy expense required for their production would be wasteful. Many functions have been suggested such as cell shape determinants, protective coats and sieves which might protect from antibodies, complement and lytic enzymes. It has also been suggested that they could be

involved in cell adhesion and recognition (Sleytr and Messner, 1988). The location of the proteins on the cell surface makes them ideal candidates for host-pathogen interactions and as potential virulence factors.

The function of the S-layer of *C. difficile* is undetermined, it is thought that it may play a role in adhesion or immune system evasion. A number of other pathogenic organisms including *Campylobacter fetus* subsp. *fetus* and *Bacillus cereus* possess SLPs which have been shown to play a role in the pathogenesis of infection. *C. fetus* subsp. *fetus* causes ovine abortion and the SLPs have been shown to be essential for colonisation and/or translocation to the placenta, but do not play a role in foetal injury (Grongono-Thomas et al, 2000). The SLPs from *Bacillus cereus* were shown to play a role in adhesion to the cell matrix (Kotiranta et al, 1998). *Lactobacillus acidophilus* is part of the normal gut flora and it has been shown that cell-surface proteins including the SLPs bind to collagen and fibronectin and may play a role in the mediation of adhesion of the bacteria to the extracellular matrix proteins (Lorca et al, 2002).

The S-layer of *C. difficile* consists of two SLPs of varying molecular mass. Early work carried out on *C. difficile* by Taekoa et al (1991), showed that there was no immunological relationship between the two SLPs, and immunoblot analysis showed the lower of the two proteins appeared to be strain specific. Earlier, Sharp and Poxton (1988), had shown that the treatment of *C. difficile* with urea released one to three cell surface proteins that reacted only with homologous antiserum. It is likely that these urea extracts included the S-layer proteins from *C. difficile*. More recently, Cerquetti et al (2000), determined that the S-layer of *C. difficile* was in fact two structurally distinct protein lattices superimposed upon one another. The outer layer

formed the square lattice, and the inner layer formed a hexagonal lattice. The study also reported that the *C. difficile* SLPs are glycoproteins. More recently, Calabi et al (2001), identified the gene coding for the *C. difficile* SLPs and showed that both proteins were derived from a single gene product through post translational processing. They showed that the amino acid sequence of the high molecular weight SLP showed some conserved regions between strains, whereas the protein of lower molecular weight showed more sequence diversity which would account for the strain specificity reported by Taekoa et al (1991) and Cerquetti et al (2000). The high molecular weight SLP from *C. difficile* showed some sequence homology to *Bacillus subtilis* amidase. One of the open reading frames identified by Calabi et al (2001), was shown to be homologous to the open reading frame of the Cw66 protein which was shown to function as an adhesin (Waligora et al, 2001) suggesting a possible role in adhesion for the SLPs. The function of the SLPs from *C. difficile* remains to be determined. Recent evidence suggests that these surface proteins play a role in the pathogenesis of *C. difficile*.

1.7.7. Fimbriae

In general, fimbriae are associated with bacterial attachment and can undergo phase and antigenic variation. However, a role for virulence has not been shown in *C. difficile*. Borriello et al (1986), showed that some strains of *C. difficile* possess fimbriae but that no correlation with toxin production, the disease state of experimental animals or adherence to the gut could be made. This may reflect the *in vitro* situation where fimbriae may not be expressed.

1.8. Laboratory diagnosis of CDAD

Laboratory diagnosis of *C. difficile* requires detection of toxin from patients with diarrhoea. Only liquid stools should be investigated to confirm a clinical diagnosis of CDAD. Formed stool samples should only be investigated for epidemiological purposes (Delmee, 2001).

1.8.1. Detection of *C. difficile* by culture methods

Early efforts to isolate *C. difficile* used selective media with agents including clindamycin hydrochloride and p-cresol (Bartlett et al 1977a; Hafiz and Oakley 1976). In 1979, George et al developed a selective medium; cycloserine, cefoxitin-fructose agar (CCFA). The medium was successful and it is the basis of this formula that is still used in many laboratories. The original formula used cycloserine at 500mg/L and cefoxitin at 16 mg/L. However, several studies indicated that the concentration of the selective antibiotics were too high and the growth of some strains were inhibited, as a consequence the concentrations were halved (Willey and Bartlett, 1979; Levett, 1985). Various methods for improving selectivity and recovery have been suggested. Delmée, (2001) states that for the majority of cases, direct plating of stools to CCFA and incubation under anaerobic conditions for 24-48 hours is a satisfactory method of recovering *C. difficile*.

Treatment of faecal samples with alcohol shock prior to plating was shown to improve recovery and enhance selectivity (Borriello and Honour, 1981). Wilson et al (1982b), showed that bile salts such as sodium chlolate and taurochlolate enhance spore recovery, by enhancing germination. Cholic acid sodium salt is much cheaper and is an as effective alternative to sodium taurocholate in selective media (Brazier,

1993). Enrichment of specimens has also been shown to enhance recovery significantly. However, the clinical significance of the small numbers of bacteria recovered by enrichment is unclear. Enrichment studies are more useful for recovery of small numbers in epidemiological studies (Riley et al, 1987).

The UK Anaerobe Reference Unit (ARU) in Cardiff currently recommends the use of cycloserine cefoxitin egg-yolk agar (CCEY) agar for the detection of *C. difficile* (Brazier, 1990). The medium contains cholic acid sodium salt, *p*-hydroxyphenylacetic acid to enhance the colony odour, lysed blood to facilitate fluorescence under ultra-violet (UV) light and egg-yolk to screen out lecithinase and lipase producers. *C. difficile* colonies are relatively easy to identify by their characteristic odour, colonial morphology and fluorescence under UV light. The fluorescence exhibited by *C. difficile* is not unique, several other *Clostridium* spp. present this characteristic (Brazier, 1993).

Gas liquid chromatography can be used if necessary to confirm the identity of cultures thought to be *C. difficile*. However, this facility is not available in most diagnostic laboratories (Levett, 1984). Biochemical test and commercial latex kits can also confirm identity, however cross-reactions with *C. sordellii* and *C. bifermentans* have been reported with latex kit tests (Bowman and Riley, 1986a).

1.8.2. Detection of *C. difficile* toxin(s)

The cytotoxic activity of *C. difficile* can be detected in faecal filtrates by challenging many different cell lines such as Vero, HeLa and HEp2 with the faecal filtrate. The cytopathic effect is a result of the activity of toxin B, which is about 1000 times more potent than toxin A (Lyerly and Wilkins, 1995). The specific cytotoxic effect is confirmed by neutralisation with *C. difficile* or *C. sordellii* antiserum. The tissue

culture assay can take between 24-48 hours and requires the upkeep of tissue culture cell lines in the appropriate facilities. However, reliable results can be obtained in around five hours if large amounts of toxin are present (Delmée, 2001).

A large number of commercial kits are available for the detection of the *C. difficile* toxins in faecal specimens. Enzyme immunoassay (EIA) kits such as Clearview™ *C. difficile* A and Triage® *C. difficile* Panel detect toxin A in faecal samples via a colour change and are designed for single use. A large number of Enzyme- linked immunosorbent assay (ELISA) kits, most of which detect toxin A only, are also available. More recently however, a number of kits such as Techlab™ *C. difficile* A and B which can detect both toxins in faecal specimens have been introduced as a result of reported outbreaks due to toxin A-B+ strains of *C. difficile* (Alfa et al, 2000).

Most of these kits have been evaluated and in general they offer good sensitivity and specificity. ELISA kits are almost as specific as tissue culture, but tend to be slightly less sensitive. The major advantage of these kits over tissue culture is their rapidity and the ease of use. The ELISA based assays are costly per test and as a result many laboratories test specimens in batches. This is however also the case with tissue culture. Single use tests are more economical for only one or two tests, but may not offer the sensitivity and specificity required.

The clinical diagnosis of *C. difficile* is difficult, as patients do not always test positive for both toxin and culture, and this, together with symptoms, are required for a confirmed diagnosis. Occasionally patients test positive for toxin only and rather more frequently patients test positive for culture only and it can be difficult to make an accurate interpretation of the results. Toxin can become inactivated in samples

that are stored at ambient temperature (Bowman and Riley, 1986b; Borriello et al, 1992b) thus, the need for careful transport and storage of samples to ensure that accurate results can be obtained needs to be emphasised. The accurate diagnosis of CDAD requires good communication between clinician and microbiologist and regular monitoring of patients. Repeat samples should be collected if necessary, and should be processed rapidly.

1.9. Typing of *C. difficile*

1.9.1. Phenotypic typing methods

Early phenotypic typing methods for *C. difficile* included the combined bacteriophage and bacteriocin method used by Sell et al (1983), and the plasmid analysis, soluble protein polyacrylamide gel electrophoresis (PAGE), immunoelectrophoresis of extracellular antigens and antibiograms methods compared by Wust et al (1982).

Since these early studies a number of phenotypic methods have been investigated. Poxton et al (1984), investigated 28 *C. difficile* isolates from an outbreak of disease in Sweden by an immunochemical method that involved PAGE analysis of ethylendiaminetetraacetic acid (EDTA) extracted surface proteins and immunoblotting. Tabaqchali et al (1984b), took a different approach and incorporated [^{35}S]methionine into cellular proteins, separated them by sodium dodecyl sulphate-PAGE (SDS-PAGE) and visualised the proteins by autoradiography.

Delmée et al (1985), developed the familiar method of serotyping, which is still used to assess and compare novel typing methods. An investigation of 315 *C. difficile*

isolates from various origins formed the basis of the serological typing scheme. Of the 315 isolates under investigation, 99% agglutinated with one of the 6 different antisera raised against *C. difficile* in rabbits, and each serogroup was designated a letter A, B, C, D F and G (Delmée et al 1985). A later comparison of serogrouping and PAGE methods showed that each of ten designated serotypes was represented by a different PAGE protein pattern and that within serotype A, 12 different protein profiles were identified (Delmée, 1986a). Delmée et al (1990a), showed that cross reactivity between the SDS-PAGE subtypes of serotype A was due to flagella and that the cross-reactivity could be suppressed by the shearing off of flagella before agglutination with the antiserum. Delmée et al (1990b), later demonstrated that the serogroup specific determinant was a strain specific surface antigen responsible for agglutination. Heard et al (1986) and Sharp (1988), showed that surface proteins were strain specific and reacted only with homologous antiserum.

Pyrolysis mass spectrometry (PMS) was used by Cartmill et al (1992) and Magee et al (1993), to investigate several outbreaks of *C. difficile* infection. PMS is rapid, it offers high through-put technique and excellent discrimination, however the equipment required for PMS analysis is extremely expensive, the results are not reproducible and cannot assign permanent designations. This method is only useful for within-batch comparisons of isolates.

The preliminary findings from the international typing study on *C. difficile* showed excellent correlation between serotyping, PAGE and immunoblot typing methods which are based on cell surface proteins (Brazier et al, 1997a).

1.9.2. Genotypic typing methods

In general, genotypic typing methods are considered to be more reproducible and to offer better discrimination. Phenotypic characteristics can vary in growth conditions and growth phase making them less reliable. For these reasons phenotypic methods are often considered inferior to genotypic methods.

The first available genotypic typing methods for *C. difficile* were restriction enzyme analysis (REA) and restriction fragment polymorphism (RFLP) both of which analyse total cellular DNA. Kuijper et al (1987), used REA to analyse only six isolates from patients with CDAD and their environment, and reported that the method warranted further investigation. Devlin et al (1987), also used REA and reported the method to offer stability, good discrimination and the REA grouping agreed with cytotoxicity status. REA and RFLP have been replaced by pulsed-field gel electrophoresis (PFGE) and more recently polymerase chain reaction (PCR) based methods which are less labour intensive and easier to perform.

Kato et al (1994), used PFGE to investigate 91 unrelated isolates, and compared the method to immunoblotting. PFGE was more discriminative than immunoblotting, however problems associated with DNA degradation and untypeability were reported. In a later study, Hyett et al (1997), used PFGE to type clinical isolates from patients with diarrhoea. Fifty-six isolates produced 18 patterns, however 19 isolates could not be typed. The untypeable isolates belonged to PCR ribotype 1 and suffered from DNA degradation. PCR ribotype 1, is the type most frequently isolated from patients in England and Wales (Brazier et al, 1997b), thus most UK isolates would not be typeable by PFGE.

PFGE is highly discriminatory, however the procedure is labour intensive, technically demanding, and the major problem associated with the technique is that a large number of *C. difficile* strains have been reported to be untypeable by PFGE. This untypeability is due to difficulties with DNA degradation and this compromises the overall value of the technique. Corkhill et al (2000), reported that the problems with the DNA degradation of some strains could be alleviated by the addition of thiourea in the electrophoresis buffer. It is thought that thiourea neutralises a derivative of Tris which is formed at the anode during electrophoresis and causes the degradation of DNA, although Klaassen et al (2002), reported that even with this modification the PFGE profiles were far from satisfactory.

McMillin and Muldrow (1992), reported that *C. difficile* could be typed by arbitrarily primed PCR (AP-PCR). The method was used to investigate 41 *C. difficile* isolates. This typing method identified nine groups, with 66% of all isolates falling into one group. AP-PCR was compared to immunobotting and it offered more discrimination Killgore et al (1994). Tang et al (1995), compared AP-PCR with immunoblotting and REA, good correlation between all methods was reported. It was reported that AP-PCR was a rapid and sensitive method that was useful for typing *C. difficile*.

Random amplified polymorphic DNA (RAPD) is a similar method to AP-PCR and was used to type ten different serotypes, a further 11 outbreak related strains and 11 unrelated strains (Barbut et al, 1993). The method distinguished each of the serotypes, and demonstrated different patterns from all the unrelated strains and identified a predominant pattern within the related strains. Barbut et al (1994), used RAPD to type 30 *C. difficile* isolates from 15 HIV positive patients in an infectious disease unit. Twenty-five isolates produced the same pattern, and the other five

produced five different patterns. AP-PCR was a rapid, effective, simple and discriminatory method for typing *C. difficile*.

A novel PCR-ribotyping method was applied to *C. difficile* by Gurtler (1993). The method involved the amplification of the spacer region between the 16S and 23S ribosomal ribonucleic acid (rRNA) genes by PCR using specific primers. The region is very heterogeneous, *C. difficile* possesses 10 copies of the rRNA genes and the intergenic space is highly variable both between strains, and within the same chromosome. O'Neill et al (1996), adapted and simplified the method described by Gurtler (1993), and it is currently the method used by the ARU in Cardiff. PCR ribotyping of 2030 isolates from a variety of sources has created a library of 116 distinct ribotypes (Stubbs et al, 1999).

Several studies have compared the genotypic methods described. Bidet et al (2000), reported that PFGE offered the best discrimination, however PCR ribotyping was superior to AP-PCR and PFGE, as it offered good levels of reproducibility, easy interpretation and it was easy to perform. A similar comparison of AP-PCR, PCR ribotyping and PFGE stated that PCR ribotyping was the method of choice (Collier et al, 1996), The major advantages of this method are rapidity, reproducibility and ease of performance.

Recently, a toxinotyping scheme has been developed by Rupnik et al (1998). This method is based on variations in the PaLoc which codes the toxin genes. The method correlated well with both serotyping and PFGE, and more recently good correlation was observed with PCR ribotyping (Rupnik et al, 2001).

The International Typing Study of *C. difficile* investigated a large number of isolates of diverse origin from the UK, Belgium, USA and Australia. More types than

previously thought were identified by many different typing methods and the study emphasised the need for a common typing scheme and a common nomenclature (Brazier et al, 1997a). The major problem with the typing of *C. difficile* is the lack of inter-laboratory reproducibility due to variation which can occur when reagents and equipment differ; variations in results can occur when different thermocyclers are used, or the procedure is performed by different personnel within the same laboratory.

1.10. Prevention and treatment of CDAD

1.10.1. Antibiotic and infection control policies

Good antibiotic and infection control policies that are regularly reviewed and adhered to are of paramount importance in any hospital. The combination of these policies is imperative to the control and prevention of hospital-acquired infections including *C. difficile* infection.

Aspects of the antibiotic policy that have implications on *C. difficile* disease include the avoidance of unnecessary antibiotic use, the reduction in use of antibiotics commonly associated with *C. difficile* and the use of narrow spectrum antibiotics whenever possible. Health care professionals should also be encouraged to communicate with microbiologists to ensure the use of the most appropriate antibiotic (Worsley, 1998).

Changes in antibiotic policies can significantly affect the number of cases of *C. difficile* disease. Impallomeoni et al (1995), reported an increase in the number of cases of *C. difficile* in geriatric wards following the recommendations by the British Thoracic Society to use cefotaxime for the treatment of community-acquired

pneumonia (British Thoracic Society, 1993). As a result the British Thoracic Society published new guidelines in 2001. A study by Ludlam et al (1999), reported a decrease in the number of cases of CDAD in a geriatric unit following the implementation of a policy restricting the use of intravenous third generation cephalosporins. A similar study also showed the reduction in cases of CDAD following the restrictive use of cefuroxime, and reported no increase in the morbidity and mortality of patients following the changes in the antibiotic policy (McNulty et al, 1997). Cartmill et al (1994), saw a reduction in cases of CDAD when they restricted the use of all antibiotics and encouraged the use of 4-quinolones instead of broad spectrum β-lactams and the use of narrow spectrum antibiotics.

At the forefront of an effective infection control policy is hand washing. Compliance with hand washing and hand disinfecting policies is generally thought to be low, and therefore hand contamination with pathogenic organisms is thought to be important in the transmission of infection. A study that monitored compliance with hand washing in Switzerland during a programme of hand hygiene promotion showed compliance with hand washing recommendations to rise from 48% to 66%. The improvements were due to increased compliance by nursing staff, but not by doctors. During this period nosocomial infections dropped from 16.9% to 9.9% (Pittet et al, 2000). In another study that surveyed the incidence of *C. difficile* six months before and six months after an education programme promoting the use of gloves when handling all body substances, the rate of *C. difficile* dropped from 7.7 in 1000 patients to 1.5 in 1000 patients. This indirectly provides evidence for transmission of *C. difficile* by hand colonisation (Johnson et al, 1990a). Mathematical modelling of *Staphylococcus aureus* transmission showed that relatively small increments in hand

washing compliance can be significant in controlling infections (Cooper et al, 1999). As well as fulfilling the need for compliance with hand washing there is a need for improvements in the efficacy of soaps and hand washes used in hospitals (Worsley, 1998).

Contamination of the hospital environment with *C. difficile* spores is well documented (and was discussed in section 1.3). *C. difficile* spores survive well in the environment and as there are few effective sporicidal agents available it is difficult to remove *C. difficile* from environmental surfaces, and it is plausible that cleaning may facilitate the movement of spores around the hospital ward. Often equipment such as commodes and lifting equipment are not subject to regular cleaning and may be a source of transmission of *C. difficile* (Worsely, 1998). The Department of Health guidelines state that commodes and hoists should be cleaned immediately after each use and that disposable equipment and instruments should be used where possible (Pratt et al, 2001). The need for regular cleaning of all hospital equipment after every use cannot be over-emphasised.

Wilcox and Fawley (2000), have shown sub-inhibitory concentrations of a number of commonly used hospital disinfectants and detergents to enhance sporulation. The effects of cleaning on the removal of *C. difficile* are unclear due to lack of efficient sporicides. However, regular cleaning of all areas and deep cleansing procedures in areas subjected to contamination should be encouraged. It is of great importance that all healthcare personnel are well informed about the epidemiology and modes of transmission of *C. difficile*. Awareness of the high rates of asymptomatic colonisation with *C. difficile* particularly in elderly patients and other high risk groups needs to be increased. Precautionary measures should be taken with all

patients and not only those with symptomatic infections. When cases of infection do develop, further spread can be prevented by isolating symptomatic patients and implementing enteric precautions including gloves, aprons and the immediate disinfecting of equipment and soiled surfaces or linen (Worsley, 1998).

It is unclear which of the many infection control strategies can help to reduce the incidence of *C. difficile*, therefore it is important that all aspects of infection control policies are implemented to prevent and control infection with *C. difficile*. Cartmill et al (1994), showed that strict infection control including staff education, increased vigilance, strict enteric precautions, cohort nursing, deep cleaning and restricted staff and patient movement together with a restriction on antibiotic use led to a significant decrease in the number of cases of *C. difficile*. Until the risk factors for *C. difficile* infection are well defined and effective control strategies are developed, education programmes, infection control and antibiotic policies should be developed, reviewed and complied with stringently.

1.10.2. Treatment of *C. difficile* infection

Since the emergence of CDAD, treatment of the condition with vancomycin or metronidazole has become well established and has remained relatively unchanged (Gerding et al, 1995). In a large number (23%) of cases of CDAD, discontinuation of the inciting antibiotic can resolve symptoms within several days and further intervention is not required (Teasely et al, 1983).

Current treatment strategies for *C. difficile* largely depends on the removal of the inciting antibiotic and where required the administration of a course of vancomycin or metronidazole. Vancomycin use is currently discouraged due to recent problems with vancomycin resistant enterococci in hospitals. Vancomycin is also considerably

more expensive than metronidazole and both offer equivalent efficacy in the removal of *C. difficile* (Department of Health/PHLS joint working group report, 1995). Guidelines for the treatment of CDAD recommend discontinuation of antibiotics with supportive therapy including fluids and electrolytes. Metronidazole should be prescribed upon microbiological confirmation of *C. difficile* if necessary. Vancomycin should be used only if metronidazole is ineffective, the patient is intolerant to metronidazole or critically ill (Fekety et al, 1997).

The major problem associated with CDAD is the high relapse and re-infection rate following treatment (Gerding et al, 1995). Relapse rates of 5-23% have been reported (Wilcox and Spencer, 1992). The only recognised treatment available is the further use of antibiotics (metronidazole and vancomycin), and this may perpetuate the condition by further diminishing the gut flora.

As the diarrhoea resolves the faecal levels of vancomycin and metronidazole fall, this could permit the germination of surviving spores and survival of vegetative cells leading to the proliferation of the organism and a subsequent relapse (Gerding et al, 1995). It has also been shown *in vitro* that the high faecal concentrations of metronidazole and vancomycin achieved during therapy have a bacteriostatic effect rather than a bacteriocidal effect on *C. difficile*, thus *C. difficile* may remain viable even after the completion of treatment (Levett, 1991).

Treatment regimes for recurrent problems include supportive therapy combined with re-treatment with prolonged and tapering courses of vancomycin or metronidazole. Recently biotherapeutic approaches to treatment of recurrent CDAD have been investigated in an attempt to reconstitute the "colonisation resistance" in the gut. Therapies have included the administration of *Saccharomyces boulardii,*

Lactobacillus spp. and "faecal transplants". Trials of such treatments have been small and have in most cases been used in combination with vancomycin and metronidazole.

A number of investigations have sought to determine the merits of treatment with *Saccharomyces boulardii. S. boulardii* has been shown to offer hamsters protection from cecitis if administered before treatment with clindamycin (Toothacher et al, 1984). Elmer et al (1987), showed that the administration of *S. boulardii* to hamsters, following exposure to clindamycin and treatment with vancomycin resulted in a lower count of *C. difficile*, lower toxin B titres and fewer animals tested positive in agglutination assays. The study suggested that *S. boulardii* may maintain colonisation resistance, could be antagonistic to *C. difficile*, and may be a useful treatment to prevent the relapses commonly associated with vancomycin therapy. A number of combined therapies including vancomycin and *S. boulardii* have been investigated. MacFarland et al (1994), reported a significantly lower risk of recurring CDAD following combination treatment with vancomycin or metronidazole with *S. boulardii*. Surawicz et al (2000), investigated patients with a history of recurrent CDAD, and determined that a high dose vancomycin therapy combined with *S. boulardii* decreased the frequency of recurring CDAD, when compared to high dose vancomycin therapy only.

However, there are concerns regarding the efficacy of treatment with *S. boulardii*. Lewis et al (1998), reported that *S. boulardii* did not prevent AAD in a study of elderly patients receiving antibiotics and highlighted the need for such therapies to be evaluated properly. The safety of *S. boulardii* in immunocompromised and

debilitated patients has also raised concerns as it can result in invasive fungal infection (Bassetti et al, 1998).

Other more radical treatments have included "faecal transplants" from healthy individuals, usually a close relative. Bowden et al (1981), treated 16 patients with PMC using faecal enemas to restore faecal flora and reported no ill effects and good convalescence. This approach offered good rates of success, however, there are concerns regarding the safety of such procedures. Tvede and Rask-Madsen (1989), treated six patients with a synthetic faecal enema following treatment with vancomycin, the enema consisted of a mixture of ten facultative aerobes and anaerobes and led to a rapid loss of *C. difficile* and patient recovery. Such findings warrant further research for treatment of patients with chronic recurrent infection. These treatments show varied success rates but have practical limitations and safety concerns. The success of novel treatments may depend on the individual, as factors such as the immune status of the patient are likely to be important when using biotherapeutic treatments. A novel soluble anionic polymer for the treatment of *C. difficile* is currently under development and a published study shows promising results. The polymer neutralises the effects of toxins and it has been used successfully in hamster experiments and has produced no ill effects in healthy human volunteers (Kurtz et al, 2001).

Successful treatment of *C. difficile* remains a challenge, due to the high rate of recurrences associated with current therapies and the difficulties associated with the treatment of complications such as perforations, toxic megacolon and ileus. Improved treatments that neutralise toxins, promote the re-establishment of

colonisation resistance and do not rely on traditional antimicrobial action are required.

THE AIMS OF THIS THESIS

1. To determine the level of asymptomatic and symptomatic colonisation with *C. difficile* in wards 5 and 6 of The Royal Victoria Hospital (RVH), Edinburgh.

2. To determine the level of isolation of *C. difficile* from the environment of the geriatric patients in wards 5 and 6, RVH, and to determine if there was any association with the level of patient colonisation.

3. To determine the significance of risk factors relating to age, gender, underlying disease, medication and medical procedures in patients colonised with *C. difficile*, with and without detectable levels of toxin in the faeces.

4. To determine the variation in the molecular masses, antigenic nature and immuno-reactivity of the S-layer proteins from *C. difficile*.

5. To assess a novel typing method based on the variation of the molecular masses of the SLPs from *C. difficile*, to compare it with PCR ribotyping and to determine which S-types and PCR ribotypes colonised patients in wards 5 and 6, RVH.

6. To relate the development of symptoms and the severity of symptoms to the S-type of *C. difficile* with which a patient is colonised.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Collection of patient information and clinical specimens

2.1.1. Subjects

Faecal specimens and patient information were collected from admissions to wards 5 and 6 of the Royal Victoria Hospital (RVH) in Edinburgh during the period July 1999 and December 2000, with the exception of the period 29/04/00 to 17/06/00 due to the illness of the research nurse. The research nurse designated to this research project obtained informed consent from all patients and with the help of the nursing staff collected all faecal and environmental samples.

2.1.2. Patient information

The research nurse collected information from patients relating to the following:

1. Patient hospital number
2. Patient age
3. Patient gender
4. The origin of the patient (from the community, a nursing home or another hospital/ward)
5. Ward and location within ward 5 or 6.
6. Diagnoses including colonic disease, leukaemia, neoplasia and episodes of diarrhoea.
7. The use of medications including antibiotic therapy, antacids, laxatives, steroids and the use of nasogastric or percutaneous endoscope gastrostomy (NG/PEG) tube feeding.

2.1.3. Faecal specimens

The aim of the study was to collect at least one faecal specimen from as many patients as possible who were admitted to wards 5 and 6, RVH at some point during their hospital stay. Specimens were collected and investigated for the presence of *C. difficile* irrespective of whether the patient was suffering from symptoms associated with *C. difficile* disease. Fresh faecal specimens were collected and transported to the laboratory as quickly as possible.

2.1.4. Environmental samples

During the period of the study, the environment of patients in wards 5 & 6, RVH was sampled using contact plates and swabs, details of which are given in section 2.3.1. These samples were investigated for the presence of *C. difficile*.

2.2. Isolation and identification of *C. difficile* from faecal specimens

2.2.1. Isolation of *C. difficile* from faecal specimens

A standard loopful (ca. 0.2g, or 6-10 x 20µl loopfuls of liquid specimen, depending on the consistency) of faecal material was plated directly to pre-reduced CCEY agar -details given in appendix 1, (Brazier, 1993). The pre-poured agar was supplied by Oxoid, Basingstoke, Hampshire and the cultures were incubated under anaerobic conditions at 37°C for 24-48 h. The CCEY agar plates were examined for growth after 24-48 h as required. A preliminary identification of *C. difficile* was made by characteristic smell, colonial morphology and chartreuse fluorescence under ultra-violet light (λ=365 nm). Several suspect colonies were sub-cultured to Columbia blood agar (Oxoid). The sub-cultures were incubated under anaerobic conditions at 37°C for 24-48 h as required.

2.2.2. Identification and storage of *C. difficile* clinical isolates

The suspect *C. difficile* sub-cultures were examined by wet and Gram's film, colony morphology, chartreuse fluorescence (λ=365nm) and characteristic smell. Confirmed isolates were inoculated to 4 ml cooked meat broth (CMB) medium (details are given in appendix 1) and incubated under anaerobic conditions at 37°C conditions for 48 h. The CMB cultures were checked for purity by wet and Gram's film and stored at room temperature. A list of all clinical isolates is given in appendix 2.

(Several atypical isolates were confirmed as *C. difficile* by gas liquid chromatography, this work was carried out by Mr R. Brown.)

2.2.3. Detection of toxins A and/or B directly in faecal specimens using a commercial ELISA kit for the detection of *C. difficile* toxin(s)

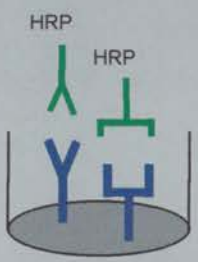
Batches of faecal samples were tested retrospectively after storage at –20°C using the Techlab™ *C. difficile* Tox A/B test kit, an ELISA for the detection of *C. difficile* Toxins A and B. In accordance with the manufacturers instructions, the faecal specimen was emulsified with 200µl of the supplied diluent and 100µl of the diluted faecal specimen was added to the supplied assay wells and the manufacturer's instructions were followed. The principle of the test kit is given in section 2.2.4 on the following page.

2.2.4. The Techlab™ *C. difficile* toxin A/B test kit

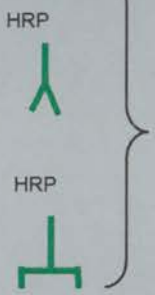
Microtitre wells coated in purified polyclonal goat antibody against toxins A and B

Conjugate = Detecting antibody:

Toxin A monoclonal mouse antibody



Toxin B polyclonal goat antibody

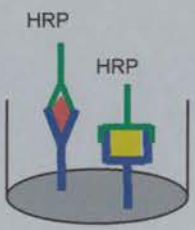


both conjugated to horseradish peroxidase (HRP)

100µl of the diluted sample containing *C. difficile* toxins A and/or B

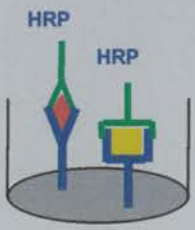
Toxin A

Toxin B



Incubation with sample and conjugate for 20 min at 37°C in a shaking incubator

Substrate



Enzyme-antibody-antigen =**colour change**
The colour was read in an Anthos 2001 plate reader at 450/620nm

2.3. Isolation and identification of *C. difficile* from the environment of wards 5 and 6, RVH, Edinburgh

2.3.1. Environmental sampling using contact plates

Environmental surfaces were sampled using 55 mm contact plates (Bibby Sterilin Ltd, Stone, Staffordshire) with pre-reduced CCEY agar (Brazier, 1993). The contact plates were placed on an inanimate surface and the agar was allowed to make contact with the surface for 5 seconds. After sampling the plates were stored in an anaerobic gas jar with an anaerobic gas generating system (Oxoid) for transport to the laboratory. The contact plates were transferred to the anaerobic cabinet and incubated at 37°C for up to 5 days. The plates were examined after 2 and 5 days as required. Suspect *C. difficile* colonies were sub-cultured to blood agar, identified and stored as described in section 2.2.2.

2.3.2. Environmental sampling using swabs

Environmental surfaces were also sampled using sterile plain cotton wool swabs (Greiner Labortechnik Ltd, Germany). The swabs were moistened in sterile distilled water before the area was sampled. The swabs were transported to the laboratory where they were inoculated to 4 ml fastidious anaerobe broth (Lab M, Bury, England) supplemented with cholic acid sodium salt (Sigma Chemicals, USA) and Modified *C. difficile* Selective Supplement (Oxoid) [further details are given in appendix 1]. The broth cultures were incubated at 37°C for 5 days under anaerobic conditions and examined for turbidity after 2 and 5 days as required. Turbid broth cultures were sub-cultured to CCEY agar for 24-48h, suspect *C. difficile* colonies were sub-cultured to blood agar, identified and stored as described in section 2.2.2.

2.4. Detection of toxin(s) from *C. difficile* isolates.

2.4.1. Growth of *C. difficile* isolates for the production of toxins

Isolates of *C. difficile* were inoculated from stored CMB cultures to 4 ml brain heart infusion/proteose peptone medium (Brettle et al, 1982) [details are given in appendix 1] for the detection of toxin, the cultures were incubated at 37°C under anaerobic conditions for 5 days.

2.4.2. Determination of the toxin–producing potential of *C. difficile* isolates from patients in wards 5 and 6, RVH

The Techlab™ *C. difficile* toxin A/B test kit, an ELISA for the detection of *C. difficile* Toxins A and B was used to detect toxin from pure *C. difficile* cultures. The 5-day cultures were centrifuged at 1500g for 15 min to remove bacteria. The culture supernatants were collected and tested for the presence of *C. difficile* toxin(s). The supernatant was diluted 1 in 5 in the supplied toxin assay diluent. Toxins A and/or B were detected by adding 100µl of the diluted supernate to the assay wells and the kit was used in accordance with the manufacturer's instructions.

2.5. Extraction of the SLPs from *C. difficile*

2.5.1. Growth of *C. difficile* cultures for extraction of SLPs

Cultures of *C. difficile* were grown in proteose peptone yeast extract (PPY) medium (Holbrook et al, 1977) pH 7.1, supplemented with sodium carbonate 0.04% w/v and cysteine hydrochloride 0.075% w/v (Poxton and Cartmill, 1982) [details are given in appendix 1]. The cultures were incubated overnight under anaerobic conditions at 37°C.

2.5.2. Treatment of *C. difficile* whole cells with guanidine hydrochloride

Bacteria were harvested from overnight cultures and washed twice in 4 ml phosphate buffered saline (PBS), pH 7.3 (Oxoid BR14-Dulbecco A tablets), by centrifugation at 1500g for 20 min. The harvested cells were re-suspended in 0.3 ml of 5M-guanidine hydrochloride and shaken for 2 h at room temperature. The bacteria were removed from the protein extract by centrifugation twice for 2 min at 16,000g. Previous work in the laboratory had determined these to be the optimal conditions for the extraction of the SLPs.

2.5.3. Dialysis of protein extracts for the removal of guanidine hydrochloride

Guanidine hydrochloride was removed from the S-layer protein extracts by dialysis against 6.25 mM Tris/HCl buffer, pH 6.8 using a Spectra/Por® Microdialyzer (Spectrum®, USA) with a membrane of 10,000 molecular weight cut off. The dialysed extracts were stored at -20°C.

2.6. Visualisation of the *C. difficile* S-layer proteins (SLPs)

2.6.1. SDS-PAGE

The surface protein extracts (ca.1 $mgml^{-1}$) were mixed with an equal amount of double strength SDS-PAGE buffer and heated in a 100°C boiling bath for 3 min. Protein extracts were run on 10% slab gels using the buffer system of Laemmli (1970), as described by Hancock and Poxton (1988). Details of all SDS-PAGE buffers are given in appendix 1.

2.6.2 Coomassie Blue Staining

The proteins separated on SDS-PAGE gels were stained with Coommasie blue stain R250 (Fisher Scientific, UK) as described by Hancock and Poxton, 1988, (details are given in appendix 1).

2.7 Analysis of the SLPs

2.7.1. Calculation of the molecular masses of the SLPs

The molecular masses of the SLPs from the *C. difficile* strains listed in appendix 2 and section 2.11.1, were calculated using Phoretix™ gel analysis 1-D software. Mark 12™ molecular weight standards (Invitrogen, USA) were used as calibrations for the calculation of molecular masses. Each isolate was designated a four-digit number based on the molecular mass in kDa of the two SLPs.

2.7.2 Immunoblotting

The Tris-glycine transfer method as described by Towbin *et al* (1979), was followed, (details of all immunoblotting buffers are given in appendix 1). The guanidine hydrochloride extracts, which were separated on SDS–PAGE, were transferred to a nitro-cellulose membrane (0.2μm pore size, Schleicher & Schuell, Germany) in Tris (0.025M), glycine (0.192M), methanol (20% v:v) buffer, pH 8.3 at 5 V and 40 mA for 18 h at 4°C.

The following procedure was carried out with gentle agitation. After washing the nitro-cellulose in Tris buffered saline (TBS: 0.02M Tris, 0.5M sodium chloride, pH 7.5) for 10 min the unbound sites on the membrane were blocked with 10 ml of 3% (w/v) gelatin in TBS for 45 min. The nitro-cellulose membrane was incubated with *C. difficile* rabbit antiserum diluted 1 in 200 in 1% (w/v) gelatin in TBS. This was

incubated at room temperature for 3 h. After washing twice for 10 min in Tween TBS (0.02M Tris, 0.5M sodium chloride, 0.025% Tween 20, pH 7.5) the membrane was incubated in anti-rabbit IgG-horseradish peroxidase (HRP) diluted 1 in 2000 at room temperature for 1 h. After two further washes in Tween TBS as above, the nitro-cellulose membrane was washed three times in distilled water. The nitro-cellulose was placed into HRP developer (details given in appendix 1) and the colour developed within 5-30 min. The development was stopped by several washes in distilled water.

2.7.3. Rabbit antiserum

Rabbit anti-sera from laboratory stocks were used. Antiserum to the following four strains of *C. difficile* were used in the study:

mprl 683 (antiserum 117)

mprl 1128 (antiserum 119)

mprl 604 (antiserum 123)

mprl 2520 [NCTC 11223] (antiserum 642).

Antisera were raised against ultra-violet killed bacteria as described by Poxton (1979). New Zealand White rabbits were injected intravenously over a five week period.

2.8. *C. difficile* S-typing based on the molecular masses of SLPs

The *C. difficile* clinical isolates collected from patients in wards 5 and 6, RVH were typed on the basis of the molecular masses of their two S-layer proteins. The S-layer proteins were extracted as described in section 2.5.1 and 2.5.2. The protein extracts were run on SDS-PAGE and Coomassie stained as described in section 2.6.2.

2.8.1. Designation of an "S-type" number to clinical isolates

The molecular masses of the SLPs were calculated as described above in section 2.7.1. Each isolate was designated a strain number (S-type) based on the four digit number assigned to each isolate.

2. 9. PCR Ribotyping of *C. difficile*

(Adapted from the method described by O'Neill et al 1996.)

2.9.1. Extraction of *C. difficile* DNA

C. difficile were sub-cultured on to fastidious anaerobe agar (Lab M, Bury, UK) supplemented with 6% horse blood (Oxoid) and incubated at 37°C in anaerobic conditions, overnight. Ten colonies of *C. difficile* were emulsified in 100µl of a 5% solution of Chelex® 100 Resin, biotechnology grade 100-2000 mesh sodium form (Biorad Laboratories, USA) and incubated in a boiling bath for 10 min. After incubation the cell debris was removed by micro-centrifugation for 2 min at 16,000g. The supernatant was retained and used as the DNA template for the PCR reaction.

2.9.2. Primers

The primers used were identical to those described by O'Neill et al (1996). The oligonucleotide primers were designed to be complementary to the 3' end (bases

1445-1446) of the 16S rRNA gene of *C. difficile* -CTGGGGTGAAGTAACAAGG and the 5' end (bases 1-20) of the 23S rRNA gene of *C. botulinum* - GCGCCCTTTGTAGCTTGACC.

2.9.3. PCR amplification mix

Amplifications were performed in 100µl reactions, containing:

10 µl template *C. difficile* DNA

1.5 units Taq DNA polymerase (Roche, Diagnostics, Mannheim, Germany).

10 µl PCR reaction buffer (100mM Tris–HCl, 15mM $MgCl_2$, 500mM KCl; pH 8.3), supplied with Taq DNA polymerase.

8 µl of a 1.25 mM solution of each deoxyribo-nucleic-triphosphate (Amersham Pharmacia Biotech inc. New Jersey, USA)

1 µl of a 50 pM solution of each primer (MWG, Germany)

The amplification program was as follows:

1 cycle:	95°C for 2 min
35 cycles:	92°C for 1.5 min
	55°C for 1 min
	72°C for 1 min
1 cycle:	72°C for 5 min.

A negative control including all reaction reagents, but excluding the DNA template was prepared for each set of reactions.

2.9.4. Concentration of PCR products

After PCR amplification the products were concentrated by evaporation on a heating block at 70°C for 90 min, or until concentrated to ca. 20µl.

2.9.5. Visualisation of the PCR Products

The concentrated PCR products were electrophoresed in 3% high resolution agarose gel (Sigma) containing 1.5% ethidium bromide at 80 volts for 2.5 h in a Scie-plas mini gel tank.

2.10. Media and Buffers

Details of all media and buffers are given in appendix 1.

2.11. Bacterial strains

C. difficile strains from the laboratory culture collection

The strains with designated serotypes and ribotypes, mprl 4196-mprl 4219 were kindly donated by Dr Jon Brazier, ARU, Cardiff, Wales. The other laboratory strains used in the S-layer protein analysis are given below.

mprl 2520	NCTC 11223
mprl 604 mprl 683	Clinical isolates from patients with antibiotic associated diarrhoea.
mprl 1128	A poorly virulent *C. difficile* clinical isolate from an infected patient-donated to the laboratory culture collection by Prof. P Borriello

Details of MPRL 4196-4219 and a list of all clinical isolates collected during the study of patients in RVH is given in appendix 2.

2.12. Statistical analysis of data

All statistical analyses (Chi-squared tests, Mann Whitney U tests and the logistic regression modelling) were carried out using SPSS software.

CHAPTER THREE

Microbiological analysis of patients and the environment of a geriatric unit in The Royal Victoria Hospital, Edinburgh.

AIMS

1. To determine the level of colonisation and symptomatic infection of patients with *Clostridium difficile* in wards 5 and 6 of The Royal Victoria Hospital (RVH), Edinburgh.
2. To determine the level of isolation of *C. difficile* from the environment of wards 5 and 6, RVH, Edinburgh.
3. To determine an association between the level of patient colonisation and the level of isolation of *C. difficile* in the environment of wards 5 and 6 RVH, Edinburgh.

RESULTS

3.1. Detection of *C. difficile* from patients in RVH

3.1.1. The study plan

Eight hundred and sixty five patients were admitted to the geriatric unit, RVH over the 17-month period (12/07/1999 to 11/12/2000). The geriatric unit consists of two wards (wards 5 and 6) with 60 beds. The unit cares for elderly patients who are debilitated and are often recovering from an illness. Many admissions are patients who have had previous care in acute care wards in other hospitals. During the study period, 500 patients aged 60 to 101 years of age were admitted to ward 5, and 365 patients aged between 56 and 100 years of age were admitted to ward 6.

The patient "mix" in ward 5 and ward 6 were different as six beds in ward 5 were used for 'respite' patients who were admitted from the community for short time periods. It was usual that these patients had no acute illness but required 24 hour nursing care.

The aim of the study was to investigate at least one faecal specimen from as many of the patients as possible who were admitted to the geriatric unit between 12/07/1999 and 11/12/2000. Over this 17-month study period a total of 1003 faecal specimens were collected from 390 (45%) of the patients admitted to the unit. Specimens from 43% (213) of patients admitted to ward 5 and 48% (177) of patients admitted to ward 6 were investigated for the presence of *C. difficile*. The specimens were investigated by culture on CCEY agar and by the Techlab™ ELISA test kit for the detection of *C. difficile* toxins A and/or B.

(NB. Unfortunately, due to the ill health of the research nurse no specimens were collected between 29/04/00 to 17/06/00).

3.1.2. Detection of *C. difficile* by culture on cycloserine-cefoxitin egg-yolk (CCEY) agar

C. difficile was isolated directly from faecal specimens using CCEY selective agar. Of the 1003 specimens from 390 patients investigated, *C. difficile* was isolated from 206 specimens, from 100 (26%) patients.

3.1.3. Detection of *C. difficile* by Techlab™ ELISA test kit for the detection of *C. difficile* toxins A and/or B

C. difficile toxin(s) A and/or B were detected directly from batches of faecal specimens which had been stored at –20°C, using the Techlab™ ELISA test kit for the detection of *C. difficile* toxins A and/or B. Of the 1003 specimens tested, *C.*

difficile toxin(s) were detected in 124 specimens, which represented 52 (13%) patients. Of these 52 patients, 18 patients tested positive for toxin(s) only and *C. difficile* was cultured from the remaining 34 patients. The results of the culture and toxin tests are summarised in table 3.1. The results in table 3.1 show the number of patients who were assigned to the following three categories based on the results of the culture and toxin tests for *C. difficile*.

1. Detection by culture test only.
2. Detection by culture and toxin test.
3. Detection by toxin test only.

Table 3.1. The culture/toxin status of patients investigated for *C. difficile* by culture and toxin detection methods.

POSITIVE RESULT IN DETECTION TEST	NUMBER (%) OF PATIENTS WITH A POSITIVE RESULT		
	WARD 5 n=213	WARD 6 n=177	TOTAL n=390
Culture only	36 (17)	30 (17)	66 (17)
Culture and toxin	12 (6)	22 (12)	34* (9)
Toxin only	10 (4.5)	8 (4.5)	18 (5)
Total	58 (27)	60 (34)	118 (30)

*Culture and toxin may not have been detected in the same specimen.

The results in table 3.1. show that *C. difficile* was detected in a total of 118 (30%) of the 390 patients investigated for the presence of *C. difficile* during the study period. Of the patients tested 17% were positive by culture only, 9% were positive by both culture and toxin tests and toxin only was detected in 5% of patients.

When the results for wards 5 and 6 were compared, the overall level of detection of *C. difficile* by culture and/or toxin from patients in wards 5 and 6 was 27% and 34% respectively. The level of detection of *C. difficile* was not a significantly different between the two wards (Chi^2 test, p=0.14). Seventeen percent of patients from both wards tested positive for *C. difficile* by culture only. However, 12% of patients from ward 6 were culture and toxin positive compared to only 6% of the patients in ward 5.

3.1.4. Incidence of diarrhoea in *C. difficile* positive patients from ward 5 and 6, RVH

The results of the culture and toxin tests from patients with diarrhoea are summarised in table 3.2. Of the patients from whom *C. difficile* was detected, 49% had diarrhoea; 40% of patients from ward 5 and 58% from ward 6. Those from whom both culture and toxin was detected had the highest levels of diarrhoea, 79% of patients in this category had symptoms compared to less than a third of those patients who tested culture-positive only.

Patients with CDAD are defined as symptomatic patients from whom both *C. difficile* and its associated toxin(s) can be detected. From this definition 4% (8/213) of patients from ward 5 and 11% (19/177) from ward 6 were determined as suffering from CDAD. However, up to a further 7% (15 patients) from ward 5 and 9% (15 patients) from ward 6 may have been suffering from *C. difficile* related symptoms, as they harboured the organism and had diarrhoea.

Table 3.2 The number of symptomatic patients in each culture/toxin category.

POSITIVE RESULT FOR DETECTION TEST	NUMBER (%) OF SYMPTOMATIC PATIENTS IN WARD 5 n=58	WARD 6 n=60	TOTAL n= 118
Culture only	12/36 (33)	8/30 (27)	20/66 (30)
Culture and toxin	8/12 (67)	19/22 (86)	27/34 (79)
Toxin only	3/10 (30)	7/8 (88)	10/18 (55)
Total	23/58 (40)	35/60 (57)	58/118 (49)

3.2. Detection of *C. difficile* in the local environment of patients in wards 5 and 6, RVH

The environment of wards 5 and 6, RVH was investigated for the presence of *C. difficile* over the duration of the 17-month study period. Environmental sampling was performed on at least monthly intervals, on 24 occasions and approximately 30 areas in each ward were sampled on each occasion. A total of 1348 environmental samples were taken during the study.

Large flat surfaces such floors, sinks and shower trays were investigated using contact plates with CCEY agar. The contact plates were stored in anaerobic conditions prior to sampling and after sampling the plates were returned to the laboratory in an anaerobic environment for incubation. Small areas such as tap handles and door handles were sampled using swabs moistened with sterile distilled water. The swabs were returned to the laboratory and then inoculated to an anaerobic enrichment medium for incubation.

3.2.1. Isolation of *C. difficile* from the environment of wards 5 and 6, RVH

Various inanimate objects from rooms within wards 5 and 6 were sampled for the presence of *C. difficile*. Surfaces and objects in the main patient bays, toilet areas, bathrooms, shower-rooms, sluice rooms, side-rooms and kitchens were all sampled. A total of 672 samples were taken from ward 5, 331 areas were sampled with contact plates and 341 with swabs. From ward 6, a total of 676 samples were taken, 344 areas were sampled with contact plates and 332 with swabs. *C. difficile* was isolated from 23% of samples taken by contact plates and 4% of samples using swabs. Details of objects, areas sampled, the method of sampling and the result are given in appendix 3.

Each ward consists of four main bays with six beds in each, and each ward had five side-rooms. Each bay had its own designated toilet area and the bathroom and shower-room facilities were shared between bays. Each of the side-rooms had its own designated toilet and bathroom facilities. In addition, each ward had a sluice-room and kitchen.

The number of samples taken and the number of areas that tested positive from each room in wards 5 and 6 is summarised in table 3.3. In ward 5, *C. difficile* was most frequently isolated from the sluice room, followed by the toilet areas associated with side-rooms. In ward 6, the most frequent isolations were from toilet areas associated with side-rooms and the side-rooms themselves. The main toilet areas and sluice room in ward 6 also had relatively high isolation levels, 17% and 15% of areas respectively. The data in table 3.3. show an overall level of isolation of *C. difficile*

from the environment of ward 5 at 7% and 20% from ward 6. Correspondingly, all rooms of ward 6 showed higher levels of isolation of *C. difficile* than the equivalent rooms in ward 5. Consistent with the overall results for wards 5 and 6, the level of isolation of *C. difficile* in the side-rooms of ward 6 was much higher than those isolation levels from the side-rooms of ward 5.

Of the areas sampled *C. difficile* was most frequently isolated from floors; 29% of all floors from which samples were taken throughout wards 5 and 6 yielded a positive culture of *C. difficile*, including one positive sample from the kitchen floor of ward 6. Of the floors sampled, the toilet floors and the sluice room floor yielded the highest isolation levels, with 40% of samples taken from toilet floors and 60% of samples from sluice room floors yielding a positive result. Other areas with relatively high isolation levels included toilet seats (17%), commodes (11%), toilet handles (8%), and three and of the five toilet cisterns tested from ward 6 gave culture positive results for *C. difficile*.

Numerous other objects were sampled and gave positive results, amongst these, the paper–towel dispenser from the ward 6 kitchen, side-room 8 in ward 6 and bay 3 in ward 6 all tested positive for *C. difficile*. Two of six windowsills in ward 6 produced culture positive results, as did 25% of the shelves and cupboard tops from the sluice rooms and bathrooms of ward 6.

Table 3.3. The number of samples taken and the number of samples positive for the culture of *C. difficile* from each room in wards 5 and 6.

	NUMBER (%) OF SAMPLES IN			
	WARD 5 NUMBER (%) OF		WARD 6 NUMBER (%) OF AREAS	
AREA	TOTAL SAMPLES	*C. difficile* POSITIVE SAMPLES	TOTAL SAMPLES	*C. difficile* POSITIVE SAMPLES
Main patient room	160	9 (6%)	138	12 (9%)
Main toilet areas	235	16 (7%)	174	30 (17%)
Bathrooms	78	3 (4%)	64	6 (9%)
Shower-rooms	41	3 (7%)	40	3 (7.5%)
Sluice rooms	60	8 (13%)	71	11 (15%)
Side-rooms: main area	53	4 (7.5%)	80	32 (40%)
Side-rooms: toilet area	43	4 (9%)	98	41 (42%)
Kitchen	2	0	11	2 (18%)
Total	672	48 (7.1%)	676	137(20%)

3.2.2. Association between environmental contamination and patient colonisation levels

There was considerable variation in the level of isolation of *C. difficile* from the environment of the different bays within the same ward. In ward 5, the level of isolation of *C. difficile* varied between 3% and 12% of the total environmental samples taken for each of the four main patient bays. The percentage of colonised patients in each of the four bays of ward 5 varied between 9% and 10%, and the percentage of colonised symptomatic patients varied between 1% and 5% over the duration of the project. In ward 6, the levels of isolation of *C. difficile* in each of the

four main patient bays varied between 0% and 16% of the total environmental samples taken. The percentage of colonised patients in the four bays of ward 6 varied between 11% and 15%, and the percentage of colonised symptomatic patients varied between 7% and 12% over the duration of the project. The percentage of colonised patients and colonised symptomatic patients in ward 6 bays is higher than in ward 5. This is consistent with a higher level of isolation of *C. difficile* from the environment of ward 6. There is however no correlation between the number colonised patients and the level of isolation of *C. difficile* from environment within the individual bays of either ward 5 or 6. It is also difficult to make any solid deductions as a significant number of patients and areas of the environment were not sampled.

Figures 3.1 and 3.2 below show the level of colonisation of patients and the percentage of environmental samples from which *C. difficile* was isolated from ward 5 and 6 over the duration of the study. The levels of patient colonisation are calculated as the percentage of positive specimens investigated during that month, and as the data show there is considerable fluctuation in the colonisation level from month to month. The levels of environmental contamination are calculated as the percentage of positive environmental samples investigated during that month.

Data from ward 5 shown in figure 3.1 does not indicate much correlation between the levels of isolation of *C. difficile* from environmental samples and the level of patient colonisation in ward 5. However, a high environmental level in November 1999 is followed by a high percentage of colonised patients in December 1999. The data from ward 6 shown in figure 3.2 are more suggestive of an association between the level of environmental contamination and patient colonisation. Between October

1999 and January 2000 there is evidence of a similar general trend in patient colonisation and environmental contamination.

Figure 3.1. The percentage of colonised patients and the percentage of environmental samples from which *C. difficile* was isolated ward 5.

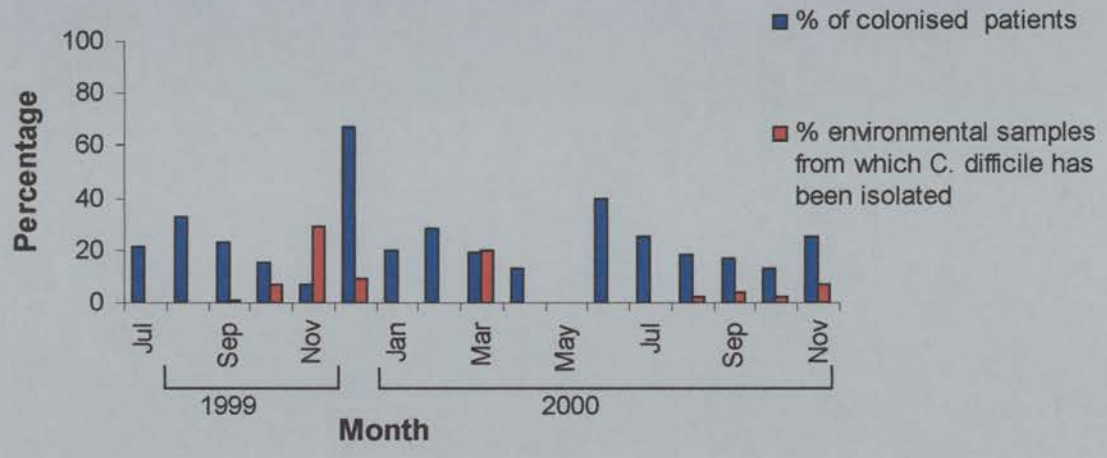
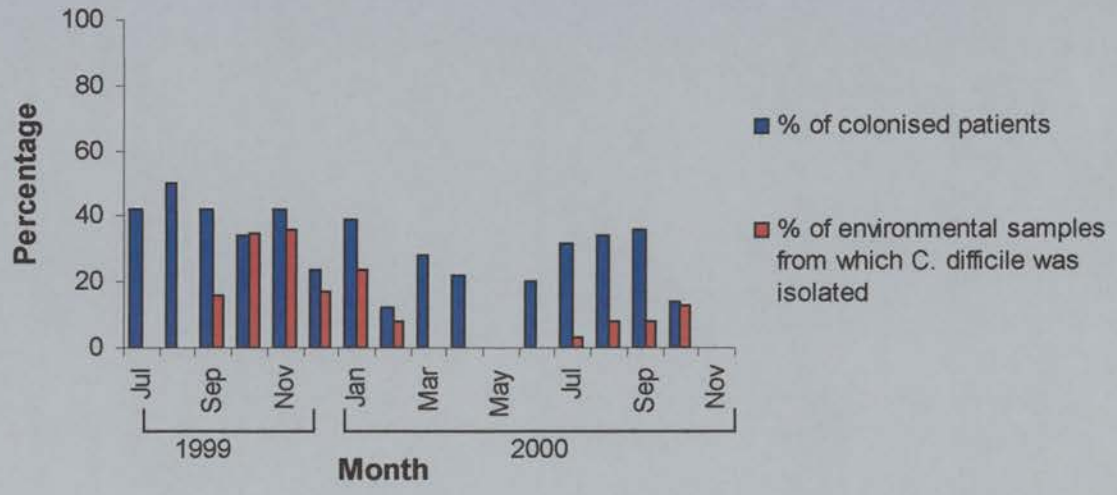


Figure 3.2. The percentage of colonised patients and the percentage of environmental samples from which *C. difficile* was isolated ward 6.



Data from several side-rooms from both wards 5 and 6 were examined. The results in figures 3.3-3.4 show the percentage of *C. difficile* positive environmental samples against the date on which the samples were taken.

Data from side-room 8 in ward 5 revealed that *C. difficile* was not isolated from this ward between 7/10/99 and 26/10/99 and that the patients resident in the side-room during this time were not colonised (data not shown). *C. difficile* was isolated from 60% of sites tested on 20/03/00 when a colonised patient occupied the side-room (data not shown). Subsequent environmental samples were negative for *C. difficile* and the patients occupying the room at these times were not colonised, thus suggesting that the presence of a *C. difficile* colonised patient leads to environmental contamination. In support of this, side-room 10, ward 5 was sampled on four occasions and on all four occasions the patient occupying the side-room was uncolonised and no *C. difficile* was isolated from the environment, this data is not shown.

Figures 3.3 and 3.4 show data from side-rooms 6 and 10 in ward 6. The data from the environment suggest high levels of environmental contamination of these side-rooms with *C. difficile*. All patients occupying these rooms at the times of sampling were colonised with *C. difficile* and the levels of isolation of *C. difficile* from the environment of side-room 6 range from 25% to 80% and side-room 10 from 0-100%.

Figure 3.3. Percentage of *C. difficile* culture positive environmental samples against the date of sample collection from side-room 6 on ward 6.

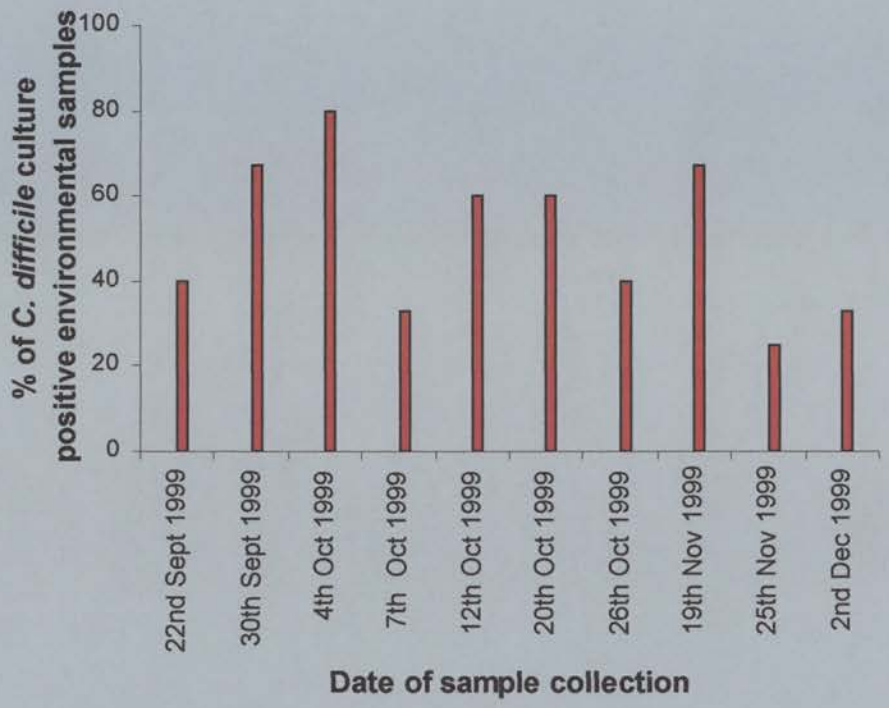
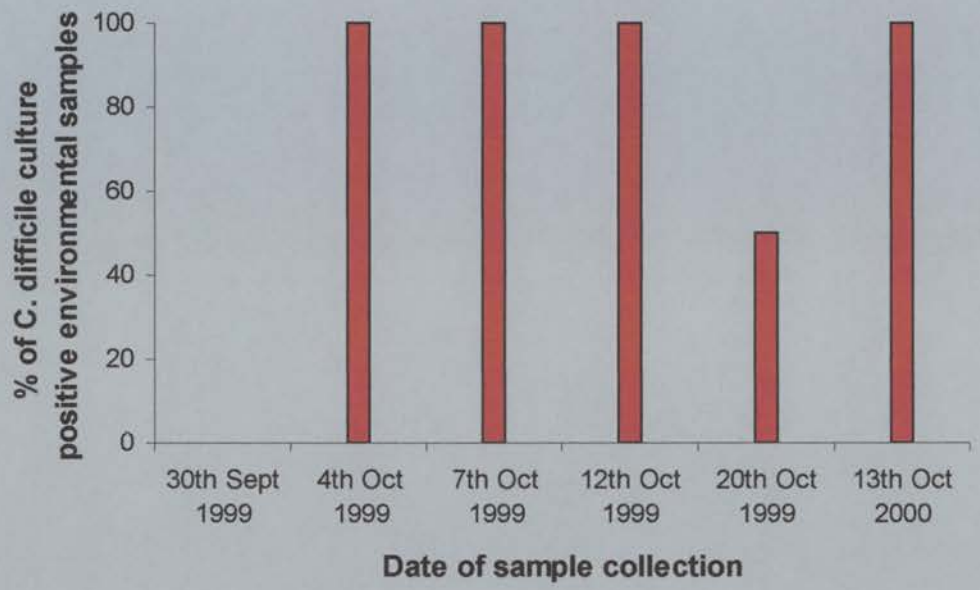


Figure 3.4. Percentage of *C. difficile* culture positive environmental samples against the date of sample collection from side-room 10 on ward 6.



The data illustrated in figures 3.3 and 3.4 suggest an association between environmental contamination with *C. difficile* and the presence of a colonised individual in a particular room.

3.3. Discussion

This study of a geriatric unit in RVH, Edinburgh investigated patients for colonisation and symptomatic infection with *C. difficile*, the study revealed a 30% incidence of colonisation with *C. difficile* in the patients investigated. The presence of *C. difficile* was detected in 27% of the patients tested in ward 5 and 34% of patients tested in ward 6.

In general, the published reported levels of isolation of *C. difficile* from different groups of hospitalised patients are highly variable. McFarland et al (1989), reported the isolation of *C. difficile* from 26% of patients in a general medical ward. Brettle et al (1982), demonstrated the isolation of *C. difficile* from 12% of patients in an Infectious Disease unit and an isolation level of 21% was shown in a combined study of patients from surgical, medical and orthopaedic wards (Johnson et al, 1990b). Gerding et al (1986), reported that 30% of a group of geriatric patients with diarrhoea harboured the organism, and in an investigation of six different Welsh hospitals, *C. difficile* was isolated from 16.5% elderly patients. Two of the Welsh hospitals investigated, reported that *C. difficile* was not isolated and that the range of acquisition from the other four hospitals was 4-24% (Brazier et al, 1999).

Approximately 30% of patients in a chronic care facility for the elderly were found to harbour *C. difficile*. Of the patients studied, 28% were toxin positive and 33% were culture positive (Bender et al, 1986). This compares well with the 27% of culture positive and 14% of toxin positive patients in RVH, it also correlates with the overall detection levels of 30% from RVH.

In contrast to the studies mentioned above, Cefai et al (1988), described levels of isolation of *C. difficile* at 7.7% and 1.9% in two separate investigations of patients in

an elderly, chronic care hospital. A similarly low isolation level was noted by Corrado et al (1990), who determined that only 4% of patients from a mixed-function ward for the elderly were positive for *C. difficile*, thus suggesting that the organism is not always endemic in elderly care wards. It should be noted that during the last decade the reported incidence of CDAD has increased exponentially, therefore the levels of *C. difficile* reported by early studies are likely to be lower than those of more recent studies.

Findings from RVH are comparable with other studies which have established colonisation levels of up to 30% in patients in elderly care units such as RVH. The published studies discussed indicate a substantial amount of variation from hospital to hospital, and as the results of the RVH study indicate, the levels of isolation of *C. difficile* also vary from ward to ward and from time to time. Differences in study methodology may also have an influence on the variation in findings between studies.

The study of patients in RVH demonstrated the variation in the level of isolation of *C. difficile* from patients over time. Levels of isolation from patients in ward 5 varied from 7-67%, and levels from patients in ward 6 varied from 0-50% per month during the study. Bender et al (1986), also noted variation in colonisation levels over time and Gerding et al (1986), also showed the identification of cases of CDAD to vary between 7 and 21 patients per month over a given time period.

Attention is drawn to the bias on sample collection from patients in RVH. Not all of the admissions to wards 5 and 6 were sampled and it is likely that specimens were easier to collect from patients with diarrhoea. As the symptomatic patients are more

likely to harbour *C. difficile* this study may overestimate the reported incidence of *C. difficile* in this geriatric unit.

From the study described 4% of patients from ward 5 and 11% from ward 6 were determined as suffering from CDAD. There were however a significant number of symptomatic patients from whom *C. difficile* was detected by culture or toxin tests, and although they did not fulfil the criteria for CDAD, their symptoms may possibly be related to infection with *C. difficile*.

The study of patients in RVH used CCEY agar to investigate the faecal samples from patients for *C. difficile*. This medium is currently recognised as the best available selective medium for the isolation of *C. difficile* and consequently it was chosen for this study. It is the medium recommended by the ARU, Cardiff, Wales and studies in our laboratory have shown it to be superior to CCFA. The medium contains cholic acid sodium salt, a more economical, but as effective alternative to sodium taurocholate (Brazier, 1993). Studies in the early 1980s showed enhanced recovery of spores from faecal samples with the use of sodium taurocholate (Wilson et al, 1982b).

The methods used in the study offered optimal recovery of *C. difficile.* The use of the best available medium and the storage of all agar plates in an anaerobic environment for a minimum of 18 hours before plating faecal material to them facilitated maximum recovery of *C. difficile*. Alcohol shock or enrichment may have recovered small numbers of bacteria that could have gone undetected by direct plating to CCEY, however any small numbers that were missed were not thought be significant and due to the highly selective nature of the CCEY agar.

Toxin was not detected in the faecal samples of 54% of patients who were colonised with *C. difficile*. It is well documented that toxin is often not present in the faeces of asymptomatic, colonised patients. This may be because the toxin it is not produced in these patients in detectable quantities, or that patients are colonised by non-toxigenic strains. It is undoubtedly the case that most of these patients did not have detectable levels of toxin in their faeces. The Techlab™ A/B kit was used as described in the manufacturer's instructions. The positive and negative controls provided with the kit were used and the results interpreted in accordance with the manufacturer's instructions. Aldeen et al (2000) and O'Conner et al (2001), carried out comparisons of the Techlab™ A/B kit with the "gold standard" tissue culture method. Both showed that the kit is highly sensitive and specific. The results from the study of patients in RVH can therefore be interpreted with confidence.

However, there are several other possible explanations, which may account for the failure to detect toxin in some of the specimens. Degradation of *C. difficile* toxins occurs rapidly. It is therefore possible that some toxin-positive specimens may have failed to give a positive result if there was only a very small amount of toxin present, which degraded during transport or storage at –20°C.

It is interesting to note that patients from whom both culture and toxin(s) were detected did not always produce both toxin and culture positive results in the same faecal specimen. This could be due to variation in the amount of toxin produced or the bacterial load in the gut over the time course of colonisation and/or infection with *C. difficile*. However, most patients who produced specimens from which only toxin was detected had prior to, or subsequently produced specimens from which culture was detected. Toxin only was detected from only 5% of all patients investigated, this

finding of patients from whom only toxin only could be detected is not unique, both Gerding et al (1986) and Cefai et al (1988), also reported small numbers of patients from who toxin only was detected. It is likely to reflect failure of the culture detection method as the presence of toxin also indicates the presence of bacteria.

Environmental sampling of the study area utilised both contact plates and swabs. Samples taken by contact plates yielded a much higher isolation level of *C. difficile* than the samples taken with swabs. In a study comparing the relative merits of contact plates and swab methods, it was stated that contact plates were the preferred method of sampling and that they were better than swabs (Buggy et al, 1983). It is therefore probably the case that the lower yields from the swabs were a result of the sampling method rather that the areas sampled.

Sampling of the environment of the wards RVH indicated that ward 6 was much more heavily contaminated that ward 5. *C. difficile* was isolated from 7% of environmental surfaces sampled in ward 5, and 20% of those surfaces sampled in ward 6. These findings compare with a study which isolated *C. difficile* from 15% of environmental surfaces in a chronic care ward for the elderly (Bender et al, 1986) and an investigation by Al Saif and Brazier (1999), which isolated *C. difficile* from 20% of inanimate objects from various hospital environments.

The areas with the highest isolation levels of *C. difficile* were toilet areas, sluice-rooms and side-rooms. As toilet areas and sluice rooms are most likely to become contaminated by faecal material infected with *C. difficile*, this finding is not surprising. Patients who were symptomatic carriers of the organism often occupied the side-rooms and therefore contamination of such an environment is inevitable.

The environmental study showed a correlation between the overall level of detection of *C. difficile* from wards 5 and 6 and the levels of colonisation in wards 5 and 6. In ward 6, 19% of patients were symptomatically colonised, compared to only 10% in ward 5, suggesting a relationship between symptomatic colonisation and environmental contamination.

The level of isolation in specific rooms was associated with the colonisation status of the patient occupying the room at that time. This outcome is supported by other studies which have shown that isolation of *C. difficile* is greater from the environment of colonised patients, and greater still from colonised symptomatic patients. McFarland et al (1989), demonstrated an isolation level of 29% from the environment of asymptomatic colonised patients, and 49% from the environment of symptomatic colonised patients. Fekety et al (1981), reported isolation levels of 2.5-2.8% from the environment were there were no known disease cases and 10% from the environment of known cases of *C. difficile* associated infection.

These studies also demonstrated frequent isolation of *C. difficile* from floors, commodes and toilets. McFarland et al (1989), isolated *C. difficile* from 36% of floors, 18% of toilets and 38% of commodes and Samore et al (1986), also isolated the organism from 48% of floors, 41% of commodes and 33% of toilets. *C. difficile* was most frequently isolated from the floor of RVH, and toilets and commodes were also frequently contaminated. The high levels of contamination of floors may be due to the initial contamination and subsequent movement of spores from place to place on the feet of staff, patients and visitors. Interestingly, McFarland et al (1986), and Samore et al 1986, isolated *C. difficile* from windowsills at levels of 30% and 38% respectively. *C. difficile* was isolated from 20% of windowsills sampled in RVH. The

organism was also isolated from shelves and cupboard tops. Areas such as these collect dust and may not be subject to cleaning as frequently as other areas, and hence the spores collect and provide a reservoir of the organism. Fekety et al (1981), reported the recovery of *C. difficile* from an intentionally contaminated surface for up to five months, demonstrating the ability of the organism to survive in the environment.

The data from RVH and other previous investigations demonstrate the widespread contamination of the environment with *C. difficile* and hence the opportunity for cross-infection. The need for regular cleaning and effective disinfectants is essential for the removal of the organism. Wilcox and Fawley (2000), published a study which found that some common hospital cleaning chemicals enhance spore production at sub-inhibitory concentrations, as a spore former, the difficulties in eliminating the *C. difficile* spores are challenging.

The findings from this study illustrate the endemic nature of *C. difficile* in a geriatric population and the degree to which their environment is contaminated. It emphasises the need for good infection control policies, the need for thorough, regular cleaning and the requirement for an effective agent against *C. difficile* spores.

CHAPTER FOUR

Risk Factors for colonisation and infection with *C. difficile*.

AIM

To determine by univariate and multivariate analysis, the statistical significance of exposure to a number of potential risk factors associated with *C. difficile* colonisation and disease.

As discussed in chapter three, faecal specimens from 390 patients admitted to wards 5 and 6, RVH, Edinburgh were investigated for the presence of *C. difficile*. Of the 390 patients investigated, *C. difficile* was detected in 118 patients by culture and or/toxin detection methods. A total of 66 patients tested positive by culture detection methods only (Cdc+), 34 by both culture and toxin detection methods (Cdt+), 18 patients by toxin methods only and the remaining 272 patients were *C. difficile* culture and toxin negative (Cdc-). For the purpose of this analysis the 18 patients who tested positive by toxin detection methods only will be disregarded due to the difficulties associated with categorising them. These toxin positive only results may have been a result of false positive ELISA result, or the failure to detect *C. difficile* in culture, this was discussed in detail in chapter three.

RESULTS

4.1. Risk factors for *C. difficile* colonisation and disease

Information relating to a number of factors that could potentially influence *C. difficile* colonisation and infection was collected from patients in wards 5 and 6, RVH. Data relating to medication used, diagnoses and several underlying diseases

were collected from patients in wards 5 and 6, RVH. This investigation focused on risk factors which may have the capacity to alter the normal gut flora, thus affecting the colonisation resistance. Previous studies have also implicated these risk factors investigated as influential on colonisation with *C. difficile* and the development of CDAD. The potential risk factors investigated are listed below.

1. Age
2. Gender
3. Underlying diseases
    a) Colonic disease (such as Crohn's disease and inflammatory bowel disease)
    b) Leukaemia
    c) Neoplasia
4. Medication
    a) Antacids
    b) Laxatives
    c) Steroids
    d) Nasogastric or percutaneous endoscope gastrostomy (NG/PEG) tube feeding.
5. Origin of the patient admitted to wards 5 and 6 (community, other hospital/ward or nursing home).
6. Antibiotic therapy[1]
    a) Any antibiotic use[2]
    b) Amoxycillin

c) Co-amoxiclav (augmentin)

d) "Other" penicillins (e.g. flucloxacillin)

e) Ceftriaxone (a third generation cephalosporin)

f) "Other" cephalosporins[3]

g) All cephalosporins (ceftriaxone and "other" cephalosporins)

h) Macrolides (erythromycin, clarithromycin)

i) Quinolones (e.g. ciprofloxacin)

j) Other parenteral [intravenous (IV)/ intramuscular (IM)] antibiotics

k) Trimethoprim

[1]All analyses was based on the use of antibiotics within the two-week period prior to the collection of a faecal specimen.

[2]Any antibiotic use- this group includes all antibiotics.

[3]"Other" cephalosporins- this group includes all cephalosporins except ceftriaxone.

NB. The number of patients exposed to each of the risk factors listed above and their *C. difficile* status in given in appendix 4.

The influence of these factors on *C. difficile* colonisation was investigated using both univariate and multivariate statistical analysis of the data. The data from all patients who tested negative for *C. difficile* (Cdc-) and from all those patients who tested positive (Cdc+ and Cdt+) for *C. difficile* was analysed to determine a one-step predictive model for colonisation and infection with *C. difficile*. The data were then analysed to determine a two-step predictive model (Starr et al, 1997), for colonisation and infection with *C. difficile*. It was hypothesised that the risk factors which influence conversion from Cdc- to Cdc+, may be different from those factors which influence conversion from the Cdc+ to Cdt+, which can induce diarrhoea. Evidence has shown that many patients become asymptomatically colonised with *C.*

difficile, and that exposure to certain risk factors facilitate this colonisation. It is also thought that asymptomatic colonised patients can become susceptible to CDAD as the result of further exposure to the same and/or different risk factors which allow production of large amounts of *C. difficile* toxin(s) in the gut.

Increasing age and antibiotic use have been implicated in both colonisation and infection with *C. difficile*. However, it is probable that a number of other factors may be influential in colonisation and CDAD. Logistic regression analysis was used to determine which specific factors influence the transition from Cdc- to Cdc+ and from Cdc+ to Cdt+.

4.2. Risk factors influencing the transition from a *C. difficile* uncolonised state (Cdc-) to a *C. difficile* colonised (with or without toxin in faeces) state (Cdc+ and Cdt+)

Data from the 272 patients who tested negative for *C. difficile* (Cdc-) and from the 100 patients who tested positive for *C. difficile* by culture and toxin detection methods (Cdc+ and Cdt+) were analysed. The data were investigated with univariate and subsequently multivariate logistic regression models.

4.2.1. Univariate analysis

Univariate analysis was used to investigate the exposure of the Cdc- patient group and the Cdc+ and Cdt+ patients to each of the potential risk factors under examination. Age and gender were forced into the model *a priori*. Increasing age is recognised as a significant factor in *C. difficile* disease, and the influence of age may be confounded by gender, as there is a higher proportion of females in the very

elderly population. Each of the factors under investigation was added to the logistic regression model on a univariate basis to determine which factors reached significance, the output from the univariate analysis is shown in table 4.1. This analysis indicated that age, laxative use, admission from another hospital and any antibiotic use were significant factors in the transition from Cdc- to Cdc+ and Cdt+. Individual antibiotics reaching significance were co-amoxiclav, macrolides and all cephalosporins. Univariate analysis showed that exposure to a number of factors may be significant in the transition from Cdc- to Cdc+ and Cdt+. Laxatives and macrolides were used in a higher proportion of the Cdc- patients, thus they are not thought to be significantly influential in the development of colonisation and disease. Many patients were exposed to a number of potential risk factors, for example many patients received more than one antibiotic, therefore, when investigating a multi-factorial disease such as *C. difficile* disease, it is necessary to consider individual factors simultaneously to adjust for confounding interactions amongst the factors under investigation. Multivariate analysis, was used in this study to adjust for confounding interactions.

Table 4.1. Results from univariate analysis of risk factors influencing the transition from Cdc- to Cdc+ and Cdt+.

RISK FACTOR	ODDS RATIO	95% CONFIDENCE INTERVALS	P-VALUE
Age *(increasing per year)*	1.04	1.01-1.08	0.02*
Gender	0.62	0.38-1.02	0.06
Neoplasia	0.63	0.27-1.51	0.30
Colonic disease	0.54	0.23-1.28	0.16
Leukaemia	2.69	0.36-19.84	0.33
Laxative use	0.58	0.37-0.93	0.03*
NG/PEG use	5.01	0.81-28.44	0.07
Steroid use	1.10	0.44-2.73	0.84
Antacid use	1.50	0.89-2.50	0.13
Source (Hospital and nursing home admissions compared to community)			<0.01*
Hospital	2.35	1.45-3.80	<0.01*
Nursing home	0.21	0.03-1.66	0.14
Any antibiotic use	2.52	1.60-4.16	<0.01*
Amoxycillin	2.39	0.93-6.11	0.07
Co-amoxiclav (augmentin)	1.69	1.03-2.79	0.04*
Other penicillins	1.46	0.52-4.07	0.47
Ceftriaxone	13.88	2.88-67.02	<0.01*
"Other" cephalosporins	7.84	1.99-30.96	<0.01*
Macrolide	3.06	1.43-6.65	<0.01*
Quinolones	1.42	0.70-2.90	0.34
Other parenteral (IV/IM) antibiotics	2.14	0.85-5.36	0.11
Trimethoprim	0.56	0.32-1.87	0.56
Cephalosporins	7.59	2.55-22.59	<0.01*

* - A significant statistical result

4.2.2. Multivariate analysis

Those factors that reached significance by univariate analysis were added one by one in a stepwise logistic regression model to adjust for any confounding interactions between these factors, the results are shown in table 4.2 with age and gender forced into the model *a priori.*

The reduced stepwise logistic regression analysis showed that increasing age, admission from another hospital or ward, exposure to any antibiotics, and cephalosporins, especially ceftriaxone were significant factors in the transition from Cdc- to Cdc+ and Cdt+. The final model of best fit determined age (O.R. 1.04, 95% C.I. 1.01-1.06), hospital admission (O.R. 1.99, 95% C.I. 1.21-3.29) and any antibiotic use (O.R. 2.20, 95% C.I. 1.32-3.64), especially the use of ceftriaxone (O.R. 11.53, 95% C.I. 1.2.03-65.46) as the significant factors influencing the transition from Cdc- to Cdc+ and Cdt+.

Forward conditional logistic regression was also applied to all of the factors under investigation. All factors were added simultaneously to the forward conditional logistic regression analysis and the results from this analysis are shown in table 4.3. This analysis indicated that increasing age, admission from another hospital and any antibiotic use, especially ceftriaxone were significant risk factors for the transition from Cdc- to Cdc+ and Cdt+. This model also identified the use of trimethoprim as being a borderline significant factor in the transition from Cdc- to Cdc+ and Cdt+. It is unlikely to be a significant factor in *C. difficile* colonisation or disease, as a larger proportion of Cdc- patients than Cdc+ and Cdt+ patients used trimethoprim.

Table 4.2. Results of stepwise logistic regression of risk factors influencing the transition from Cdc- to Cdc+ and Cdt+.

FACTOR	ODDS RATIO	95% CONFIDENCE INTERVALS	P-VALUE
Age (increasing per year)	1.04	1.01-1.08	0.02*
Gender	0.62	0.38-1.02	0.62
Source (Hospital and nursing home admissions compared to community)			<0.01*
Hospital	2.35	1.45-3.80	<0.01*
Nursing home	0.21	0.03-1.66	0.14
Laxative use	0.63	0.39-1.03	0.06
Any antibiotic use	2.51	1.52-4.11	<0.01*
Co-amoxiclav (augmentin)	1.02	0.55-1.88	0.96
Cephalosporins	6.02	1.87-19.40	<0.01*
ceftriaxone	11.53	2.03-65.46	<0.01*
"Other" cephalosporins	3.24	0.66-15.9	0.15
Macrolides	1.41	0.59-3.38	0.44

* - A significant statistical result

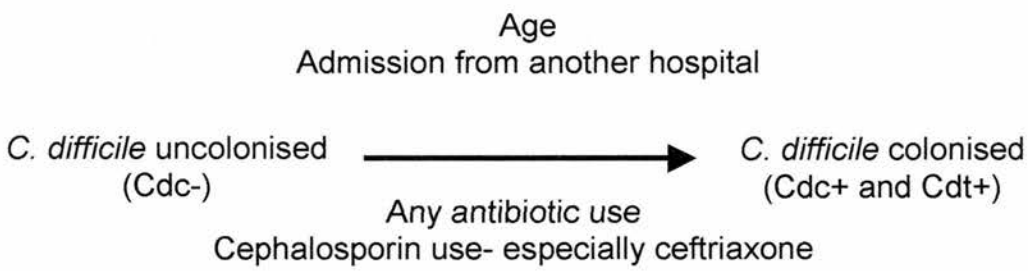
Table 4.3. Results of forward logistic regression of factors influencing the transition from Cdc- to Cdc+ and Cdt+.

FACTOR	ODDS RATIO	95% CONFIDENCE INTERVALS	P-VALUE
Age (increasing per year)	1.04	1.00-1.08	0.03*
Hospital source	1.95	1.18-3.23	<0.00*
Any antibiotic use	2.51	1.49-4.22	<0.00*
Ceftriaxone	12.51	2.21-70.69	<0.00*
Trimethoprim	0.37	0.14-1.00	0.05*

* - A significant statistical result

As described, logistic regression modelling was used to determine a one-step predictive model for factors which could significantly influence the transition from Cdc- to Cdc+ and Cdt+. The proposed predictive model is illustrated in figure 4.1.

Figure 4.1. The proposed one-step predictive model for Cdc- transition to Cdc+ and Cdt+.



As discussed in section 4.1, it has been suggested that *C. difficile* colonisation and infection may occur as a two-step process. The data were analysed using the logistic regression modelling methods described above, to determine significant factors which influence each transition in the hypothesised two-step disease process.

4.3. Risk factors influencing the transition from a *C. difficile* uncolonised (Cdc-) to *C. difficile* colonised (Cdc+) state

Data from the 272 patients who tested negative for colonisation with *C. difficile* and from the 66 culture positive only patients was analysed. The data were investigated by univariate and subsequently logistic regression modelling.

4.3.1. Univariate analysis.

Univariate logistic regression was used to analyse the exposure of Cdc- and Cdc+ patients to each of the risk factors under investigation. As before age and gender were forced into the model *a priori.* Each of the factors under investigation was added to the logistic regression model on a univariate basis to determine which risk factors reached significance and the results of the univariate analysis are shown in table 4.4. This analysis indicated that gender, admission from another hospital, any antibiotic use, macrolides and cephalosporins, especially ceftriaxone may be important in the transition from Cdc- to Cdc+.

Table 4.4. Results from univariate analysis of risk factors influencing Cdc- transition to Cdc+.

FACTOR	ODDS RATIO	95% CONFIDENCE INTERVALS	P-VALUE
Age (increasing per year)	1.03	0.99-1.07	0.19
Gender	0.56	0.31-0.97	0.05*
Neoplasia	0.81	0.32-2.05	0.65
Colonic disease	0.74	0.29-1.86	0.52
Leukaemia	0.16	0.58-31.87	4.29
Laxative use	0.63	0.36-1.10	0.11
NG/PEG	3.36	0.45-24.99	0.24
Steroid use	0.69	0.20-2.42	0.56
Antacid use	0.39	0.71-2.41	1.31
Source			<0.01*
(Hospital and nursing home admissions compared to community)			
Hospital	2.40	1.37-4.19	<0.01*
Any antibiotic use	1.87	1.08-3.23	0.03*
Amoxycillin	0.72	0.15-3.39	0.68
Co-amoxiclav (augmentin)	1.31	0.72-2.38	0.38
Other penicillins	1.76	0.59-5.29	0.31
Ceftriaxone	11.84	2.21-63.50	<0.01*
"Other" cephalosporins	2.89	0.45-18.03	0.26
Macrolides	3.17	1.34-7.56	<0.01*
Quinolones	1.10	0.45-2.66	0.84
Other parenteral (IV/IM) antibiotics	0.97	0.26-3.62	0.94
Trimethoprim	0.72	0.24-2.17	0.56
Cephalosporins	5.69	1.66-19.57	<0.01*

* - A significant statistical result.

NB. Odds ratios were omitted for patients admitted from a nursing home as they were non-significant and there were very small numbers in the group.

4.3.2. Multivariate analysis

Those factors, which showed significance in the univariate analysis, were applied one by one in a stepwise logistic regression model, and age and gender were forced into the model *a priori*. The results from this reduced logistic regression model are shown in table 4.5. The analysis showed that admission from another hospital, any antibiotic use, especially cephalosporins and in particular ceftriaxone use were significant factors influencing the transition from Cdc- to Cdc+.

The final model with the best fit showed that factors reaching significance were admission from another hospital (O.R. 2.15, 95% C.I. 1.20-3.85), the use of ceftriaxone (O.R. 16.57, 95% C.I. 1.80-151.86).

Table 4.5. Results of reduced stepwise logistic regression of risk factors influencing the transition from Cdc- to Cdc+.

FACTOR	ODDS RATIO	95% CONFIDENCE INTERVALS	P-VALUE
Age (increasing per year)	1.03	0.99-1.07	0.19
Gender	0.56	0.32-1.00	0.05
Source (hospital admissions compared to community)			<0.00*
Hospital	2.40	1.37-4.19	<0.00*
Any antibiotic use	1.80	1.02-3.17	0.04*
Cephalosporins	6.03	1.52-23.94	0.01*
Ceftriaxone	16.57	1.81-151.86	0.01*
Macrolide	2.40	0.77-5.42	0.15

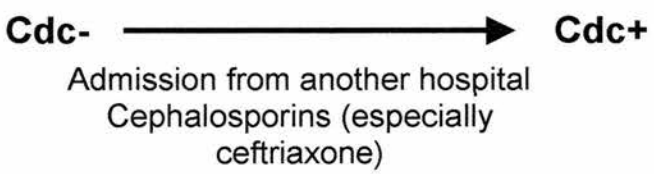
*- A significant statistical result.

NB. Odds ratios were omitted for patients admitted from a nursing home as they were non-significant and there were very small numbers in the group.

Forward conditional logistic regression modelling was also applied to the data. All factors under investigation were added to the model simultaneously and the resulting analysis showed that the factors reaching significance were admission from another hospital (O.R. 2.30, 95% C.I. 1.31-4.41) and the use of ceftriaxone (O.R. 18.95, 95% C.I. 2.12-169.31).

The analysis described above determined factors which are important for the first step of the proposed two-step predictive model. The model is illustrated in figure 4.2.

Figure 4.2. Factors influencing the first step of the proposed two-step predictive model.



4.4. Factors influencing the transition from a *C. difficile* colonised (Cdc+) to *C. difficile* colonised with detectable toxin in faecal samples state (Cdt+).

The data from the 66 culture positive only patients (Cdc+) and the 34 culture and toxin positive patients (Cdt+) was investigated using the univariate and multivariate analysis.

4.4.1. Univariate analysis

Each of the factors under investigation was added to a logistic regression model on a univariate basis to determine which factors reached significance in the transition

from Cdc+ to Cdt+. Age and gender were forced into the model *a priori*. The results are shown in table 4.6. Factors reaching significance were any antibiotic use, amoxycillin, other parenteral (IV/IM) antibiotics and "other" cephalosporins.

Table 4.6. Results from univariate analysis of risk factors influencing Cdc+ transition to Cdt+.

FACTOR	ODDS RATIO	95% CONFIDENCE INTERVALS	P-VALUE
Age	1.04	0.98-1.10	0.23
Gender	1.42	0.58-3.50	0.45
Neoplasia	0.37	0.04-3.31	0.37
Colonic disease	0.23	0.03-2.09	0.19
Laxative use	0.78	0.32-0.19	0.57
NG/PEG	2.76	0.36-21.50	0.33
Steroid use	3.06	0.60-15.55	0.18
Antacid use	1.32	0.53-3.31	0.57
Source (hospital and nursing home admissions compared to community)			0.94
Hospital	1.04	0.44-2.49	0.93
Any antibiotic use	3.05	1.15-8.12	0.03*
Amoxycillin	8.80	1.66-46.60	0.01*
Co-amoxiclav (augmentin)	2.04	0.85-4.85	0.11
Other penicillins	0.42	0.05-3.82	0.44
Ceftriaxone	1.83	0.44-7.61	0.40
"Other" cephalosporins	7.36	1.35-40.35	0.02*
Macrolide	0.91	0.28-2.97	0.87
Quinolones	1.87	0.55-6.33	0.31
Other parenteral (IV/IM) antibiotics	5.37	1.17-24.64	0.03*
Trimethoprim	1.30	0.26-6.52	0.75
Cephalosporins	2.29	0.66-8.01	0.19

*- A significant statistical result

NB. Odds ratios were omitted for patients admitted from a nursing home and patients with leukaemia as they were non-significant and there were very small numbers in the group.

4.4.2. Multivariate analysis

The factors that were significant by univariate analysis were added one by one to a stepwise logistic regression model. Age and gender were forced into the model *a priori*, the results from the reduced logistic regression analysis is shown in table 4.7.

Table 4.7. The results of stepwise logistic regression of factors influencing the transition from Cdc+ to Cdt+.

FACTOR	ODDS RATIO	95% CONFIDENCE INTERVALS	P-VALUE
Any antibiotic use	3.02	1.15-7.92	0.02*
Amoxycillin	6.13	1.16-32.37	0.03*
"Other" cephalosporins	5.74	1.02-32.34	0.05*
Parenteral (IV/IM) antibiotics	2.95	0.59-14.60	0.19

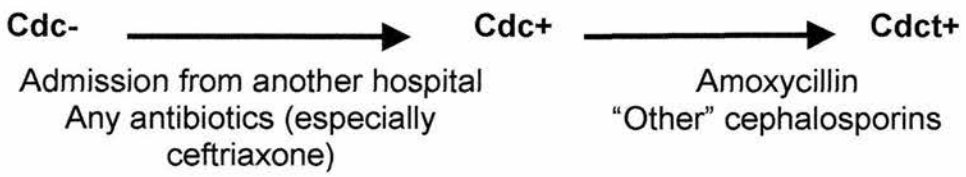
* - A significant statistical result.

The final model of best fit determined amoxycillin (O.R. 8.72, 95% C.I. 1.66-45.86) and "other" cephalosporin use (O.R. 7.27, 95% C.I. 1.34-39.65) as significant factors influencing the transition from Cdc+ to Cdt+.

Forward conditional logistic regression was applied, all factors were added simultaneously to the model. This model also determined amoxycillin (O.R. 0.12, 95% C.I. 0.02-0.60) and "other" cephalosporin use (O.R. 0.14, 95% C.I. 0.03-0.75) as significant factors in the transition from Cdc+ to Cdt+.

The analysis described above has determined a two-step predictive model for the colonisation and infection with *C. difficile*. The proposed model is shown in figure 4.3.

Figure 4.3. A two-step predictive model for *C. difficile* colonisation and infection.



This logistic regression analysis provides good evidence that colonisation and infection with *C. difficile* is a two-step process and that each of the two-steps is influenced by different factors. The initial one-step predictive model showed that increasing age was a significant risk factor. Cdt+ patients were shown to be significantly older than the Cdc+ patients ($p=0.01$, Mann Whitney U test), the mean age of Cdt+ patients was 85.26 compared to 82.23 for Cdc+ patients. Age did not appear as a significant factor in the logistic regression modelling of either of the two individual steps, however it was important in the overall disease process.

4.5. Discussion

CDAD in hospitalised patients is well recognised as a multi-factorial disease which has been associated with a number of predisposing risk factors. In 1978, Bartlett et al, identified *C. difficile* as a major cause of AAD, thus the association of CDAD with exposure to antibiotics is well established. However, not all patients who undergo antibiotic therapy develop CDAD, or even become colonised with *C. difficile*. This can be explained partly by the varying degrees to which different antibiotics influence colonisation with *C. difficile* and CDAD. It is also is well established that other factors relating to underlying diseases, medication, medical procedures and the general health of the patient are likely to be influential in the development of *C. difficile* disease.

C. difficile colonisation and disease is predominately associated with elderly patients. A number of studies have provided statistical evidence that increasing age is a significant risk factor for CDAD. Aronnson et al (1985), surveyed the incidence of *C. difficile* as the cause of antibiotic associated diarrhoea in Swedish patients aged from <1 year to 101 years of age. The study determined a higher incidence of CDAD in patients over 70 years old. In another study, records from all *C. difficile* positive patients in Sweden in 1995 were investigated. The study determined that there was a ten-fold higher incidence of *C. difficile* in the group aged 60 to 93, and that more cases occurred in the geriatric wards than in the other types of ward (Karlstrom et al, 1998). Increasing age was also a significant factor in *C. difficile* carriage and diarrhoea in a study of 399 patients aged 18 to >75 in a general medicine ward over an 11-month period (McFarland et al, 1990).

Age and the use of antibiotics are well-established risk factors for *C. difficile* colonisation and disease. However, it is clear there must be other predisposing factors for the disease, as many hospitalised elderly patients receive antibiotics and do not become colonised with, or develop *C. difficile* disease.

The major risk factors which predispose patients to colonisation and infection with *C. difficile* have been the subject of numerous investigations. Studies have investigated the possible influence of a range of diagnoses, medical procedures and underlying diseases, of which many have been significantly associated with CDAD (McFarland et al, 1990; Bignardi, 1998; Buchner and Sonnenberg, 2001). Comparison of these studies is difficult as the patient groups, the methods of data collection and analysis is variable between studies.

This study of patients in RVH, Edinburgh investigated an endemic setting where all patients subject to analysis were investigated for the presence of *C. difficile*. Comparisons were made between *C. difficile* negative patients (Cdc-), *C. difficile* culture positive only patients (Cdc+) and *C. difficile* culture and toxin positive patients (Cdt+). Initial analysis considered CDAD as a one step process. Subsequent analysis considered CDAD as a two-step disease process. It was hypothesised that *C. difficile* disease occurs as a two-step disease process (Starr et al, 1997). The proposed two-step model suggests that patients undergo the transition from a *C. difficile* culture negative state (Cdc-) to an often asymptomatic *C. difficile* culture positive state (Cdc+), with no detectable toxin in the faeces. The patient may remain in this asymptomatic state, or the patient may undergo the transition from Cdc+ to *C. difficile* culture and toxin positive (Cdt+).

The aim of this study was to determine which of the risk factors investigated significantly influenced the overall disease process and each of the two-steps in the model. The study investigated risk factors that had been implicated by other workers and that focused on medications, medical procedures and underlying diseases which might affect the endogenous gut flora therefore reducing colonisation resistance and making the patient susceptible to *C. difficile* colonisation and disease. It was anticipated that the study would provide useful information regarding the risk factors which allow colonisation with *C. difficile,* followed by the factors that allow or enhance toxin production in high levels in the gut.

When the disease was considered as a one-step process and the transition from Cdc- to Cdc+ and Cdt+ was investigated, the analysis determined that age, admission from another hospital, any antibiotic use, especially cephalosporins, and in particular ceftriaxone, were significant factors in the transition. These findings correlated well with other studies. Univariate analysis also implicated co-amoxiclav. However, multivariate analysis failed to find this significant, and as co-amoxiclav has been reported as having a low association with *C. difficile*, it is likely that it is not a significant factor.

When the two-step model was investigated, the analysis determined admission from another hospital, any antibiotic use, and the use of ceftriaxone (a third generation cephalosporin) as the factors which significantly influenced that transition from Cdc- to Cdc+. When the second transition from Cdc+ to Cdt+ was investigated the factors which significantly influenced the transition were amoxycillin and the "other" cephalosporins (first and second-generation cephalosporins). These findings indicate

that different risk factors are significant in each of the two-steps in the proposed model, thus the findings support the proposed model.

Almost all antibiotics have been associated with *C. difficile*, however the evidence suggests that certain antimicrobial agents are more frequently associated with the disease than others. Much of the evidence implicates cephalosporin use as the most potent inducer of CDAD, in a study of 130 patients diagnosed with *C. difficile* diarrhoea cephalosporins were shown to be the most frequently implicated antibiotic (Silva et al, 1984). Cephalosporins were also shown to be 40 times more likely to induce *C. difficile* than narrow spectrum penicillins (Arronson et al, 1985). In 1993 the British Thoracic Society recommended the used of cephalosporins to treat community acquired pneumonia. As a result of the implementation of this practice Impallomeni et al (1995), showed a direct correlation between the use of cefotaxime and the number of cases of *C. difficile* diarrhoea in a geriatric unit. The study indicated that up to one in every five users of cefotaxime would develop *C. difficile* associated diarrhoea. This study suggested that cefotaxime should be used as a last resort in geriatric patients.

It is interesting that the investigation from patients in RVH shows that a third generation cephalosporin, ceftriaxone is associated with *C. difficile* colonisation and that the "other" (first and second generation) cephalosporins are associated with toxin production. The model also implicates amoxycillin in the transition from Cdc+ to Cdt+. This is not surprising, as amoxycillin and ampicillin have been strongly associated with CDAD in previous investigations. Silva et al (1984), found that 41 of 130 cases of CDAD had been exposed to ampicillin. Bartlett et al (1981), also

showed that of 329 cases of CDAD reviewed that 109 had been exposed to ampicillin and amoxycillin.

It can be hypothesised that different antibiotic agents have different effects on both *C. difficile* and the normal gut flora and may therefore explain the finding from the study in RVH. Both the cephalosporins and some penicillins (such as ampicillin and amoxycillin) are broad spectrum agents and therefore their deleterious effect on the colonic flora would be considerable, thus reducing the colonisation resistance in the gut and increasing susceptibility to *C. difficile.*

Borriello and Barclay (1986), have shown that the colonic flora is inhibitory to *C. difficile* growth and toxin production. It has also been shown that *Bacteroides* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *Streptococcus* spp. inhibit *C. difficile* (Rolfe et al, 1981).

A study by Canawati, (1992) showed that cefotaxime acted in synergy with its metabolite desacetylcefotaxime to increase activity against *Bacteroides fragilis.* This illustrates the intricate nature of antibiotic activity in the complex environment of the gut, suggesting that there is much to be understood about the actions of antibiotics on the gut flora.

Less is known about the effect of antibiotics on *C. difficile* itself. It is reasonable to suggest that certain antibiotics could up-regulate virulence factors and growth. Relatively little research has focused on this area, however several studies have investigated the effect of antibiotics on toxin production. Increases in the production of enterotoxin when *C. difficile* is grown in sub minimum inhibitory concentrations of antibiotics including clindamycin and cephaloridine have been shown (Honda et al, 1983). Freeman and Wilcox (1999), stated that in their study that cefotaxime,

ciprofloxacin and piperacillan-tazobactam did not have any effect on the growth or toxin production by *C. difficile*. As relatively little is known about the virulence factors of *C. difficile* and the mechanisms of disease it is difficult to speculate how factors such as antibiotics could affect *C. difficile in vivo*.

The significance of admission from another hospital is likely to be explained in the same way as the association of the length of stay with *C. difficile*. Length of stay has been implicated as a risk factor for *C. difficile* by several workers (MacGowan et al, 1995 and Rudensky et al, 1993) However, Shek et al (2000) and Bignardi (1998), showed that length of stay was not a significant risk factor in *C. difficile* disease. Bignardi (1998), suggests that most studies have failed to address this factor correctly, and that length of stay is confounded by other factors such as antibiotic use. It is also suggested that extended length of stay is a result of *C. difficile* infection rather than the cause. Although length of stay was not investigated in patients in RVH, the significantly higher incidence of colonisation in patients admitted from another hospital/ward would indicate that exposure to the hospital environment is a risk factor for colonisation. In agreement with Bignardi (1998), McFarland et al (1990), showed that length of stay was significantly longer both in asymptomatically colonised patients and in patients with diarrhoea. McFarland et al (1990), suggested that the significant association with the length of stay was by virtue of the fact that increased length of stay provides more opportunity for a patient to be exposed to potential risk factor such as antibiotics, the hospital environment and health care workers. Thus, the patients who had been hospitalised elsewhere before admission to RVH would have had more opportunity for exposure to risk factors, and were thus more likely to be colonised than those admitted from the community.

As discussed, age is a well-established risk factor for *C. difficile* disease. The study of patients in RVH showed that Cdt+ were significantly older that Cdc+ (however Cdc+ were not significantly older than Cdc-) suggesting that even within the geriatric population older patients are more likely to develop the CDAD. The risks associated with age are likely to be a result of numerous intrinsic host factors such as general debilitation and a less efficient immune system. Borriello and Barclay (1986), showed that faecal emulsions from elderly patients were less inhibitory against *C. difficile*, suggesting a decrease in colonisation resistance, thus requiring less compromise before becoming susceptible to *C. difficile*.

Age is obviously a factor which cannot be controlled, however the antibiotic and medical procedures to which the patients are exposed can be controlled. An increased knowledge of the disease process, significant risk factors and the effects of these factors on the mechanisms which cause *C. difficile* disease are needed in order to develop effective control and prevention strategies.

Starr et al (1997), proposed a "herd immunity" model. The model suggests that within a ward ("herd") there is an overall state of resistance to *C. difficile*. However, as the number of resistant patients in the ward drops below a critical level, then cases of *C. difficile* will occur. It is thought that the use of antibiotics (and other factors) can reduce the number of resistant patients, thus shifting the equilibrium, and with more susceptible patients an outbreak may occur. This model reinforces the need for control over significant influential factors, and it suggests that careful case-mix could reduce the incidence of *C. difficile* by maintaining a resistant "herd".

The study of patients in RVH provides evidence for the two-step predictive model and it indicates that different risk factors are significant for each of the two-steps to

occur. CDAD is clearly a complex disease in which a number of risk factors play a role. However, until more is understood in relation to how *C. difficile* causes disease then results from epidemiological studies such as this are difficult to interpret and exploit to develop effective prevention and control strategies.

CHAPTER FIVE

An investigation of the variation and antigenic nature of the surface layer proteins of *C. difficile*.

AIMS

1. To extract the surface layer proteins (SLPs) from *C. difficile* isolates and to determine the variability of the molecular masses of the SLPs amongst isolates of *C. difficile*.
2. To determine the antigenic nature and immuno-reactivity of the SLPs of *C. difficile*.

RESULTS

5.1. Extraction of SLPs from *C. difficile* by treatment of whole cells with 5M guanidine hydrochloride

The SLPs of twenty-eight *C. difficile* isolates from the laboratory culture collection were investigated. Twenty-four of the isolates investigated (mprl 4196 to 4219) represented a different serotype or ribotype of *C. difficile*. The remaining four isolates (2520 [NCTC 11223], 1128, 604, 683) were those isolates to which antisera was raised in four different rabbits. (Details relating to the 28 laboratory isolates are given in chapter 2.11 and appendix 2). The *C. difficile* isolates were cultured and the SLPs were extracted by treatment with 5M guanidine hydrochloride. Treatment of whole *C. difficile* cells with guanidine hydrochloride was shown to produce two major and several minor protein bands on SDS-PAGE. This characteristic banding

pattern is indicated in figure 5.1(a). The two major protein bands indicated on Figure 5.1(a) and (b) are characteristic of the SLPs described by Kawata et al, (1988).

5.2. Variation of the molecular mass of the SLPs among *C. difficile* isolates of different serotypes and ribotypes.

Figures 5.1(a) and (b) show the high degree of variation in the molecular mass of both of the SLPs amongst the *C. difficile* isolates investigated. The larger of the two major proteins varies in molecular mass from 50-57 kilo Daltons (kDa) and the smaller protein from 35-48 kDa. Several minor proteins are visible in most of the extracts from the isolates tested. One of the minor proteins appears to be conserved, is common to most isolates and has a molecular mass of 70 kDa, it is not thought to be one of the SLPs.

Each of the twenty four isolates of different serotype or ribotype (mprl 4196-4219) was designated a 4-digit "S-type" number based on the molecular masses of the two SLPs; e.g. 5336, where 53 is the molecular mass of the heavier protein and 36 is the molecular mass of the lighter protein, in kDa.

The isolates were grouped on the basis of this "S-type" number, and this is shown in table 2. The twenty-four isolates investigated were assigned to a total of 16 groups.

Figure 5.1(a) and (b). SDS-PAGE of S-layer proteins from *C. difficile* isolates of different serotypes, ribotypes (mprl strains 4196-4219; 4217 not shown) and from laboratory isolates 2520, 1128, 604 and 683.

Figure 5.1(a)

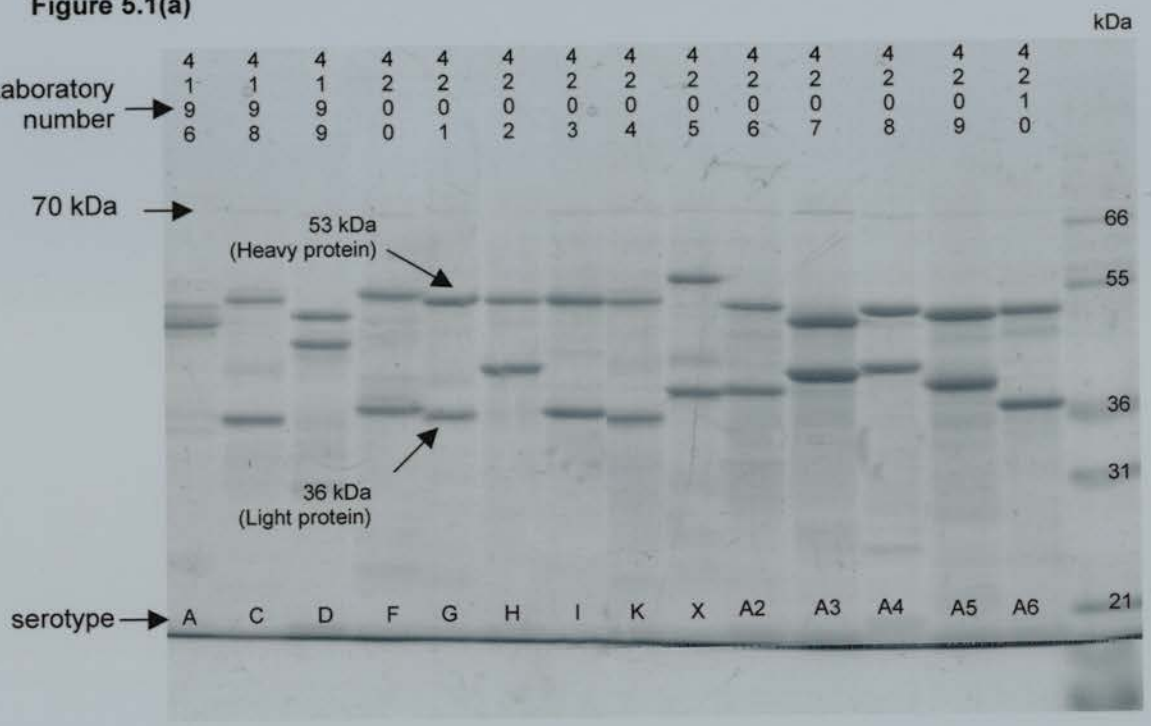


Figure 5.1(b)

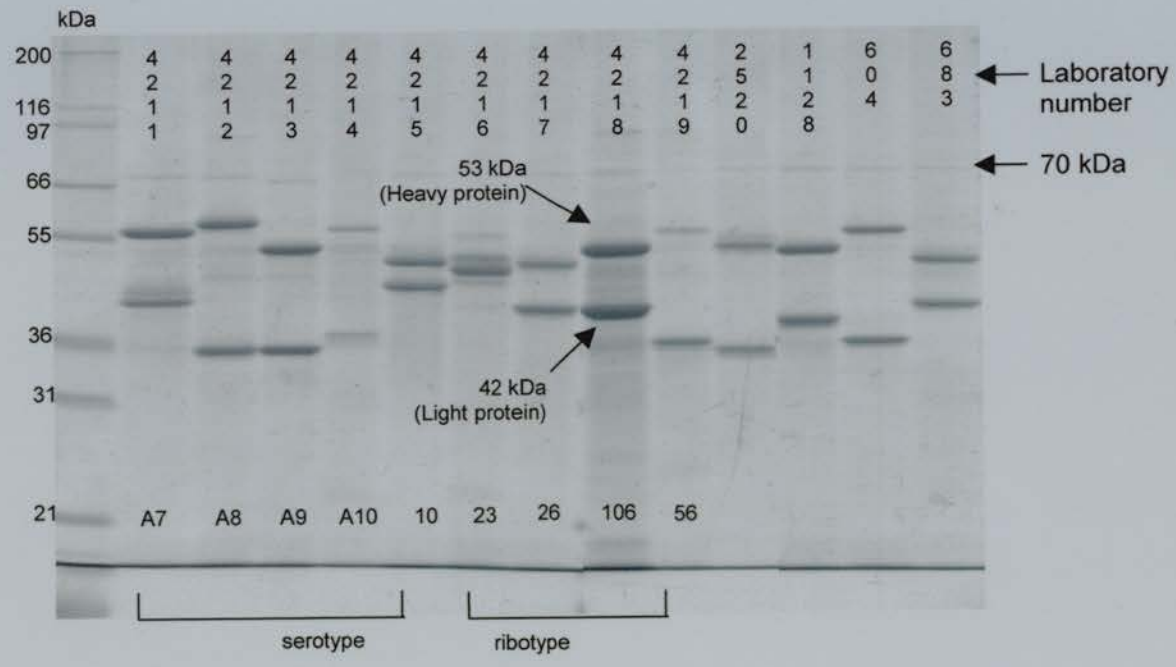


Table 5.1. Twenty-four *C. difficile* isolates of different serotype and ribotype grouped on the basis of their "S-type" number.

S-type number	Serotype (Laboratory number)			Ribotype
51 48	A (4196)			23 (4216)
50 41	A3 (4207)			26 (4217)
50 45	D (4199)			10 (4215)
51 37	A6 (4210)			
51 40	A5 (4209)			
51 42	A4 (4208)			
52 39	A2 (4206)			
53 35	A9 (4213)			
53 36	C (4198)	G (4201)	K (4204)	
53 37	I (4203)			
54 37	F (4200)			
53 42	H (4202)			106 (4218)
56 43	A7 (4211)			
57 35	A8 (4212)			
56 37	B (4197)	A10 (4214)		56 (4219)
56 38	X (4205)			

5.3. The antigenic nature and immuno-reactivity of the *C. difficile* SLPs.

Western blots were carried out using four different antisera. Each antiserum was raised against a different *C. difficile* isolate from the laboratory culture collection (mprl 604, 683, 1128, 2520). Each antiserum was tested for reactivity against the SLPs from the isolate to which the antiserum was raised and to the SLPs of six other isolates. The results from the Western blots are shown in Figures 5.2-5.5. and are discussed below.

Figure 5.2(a) shows the Coomassie stained SDS-PAGE of the SLPs from isolate 683 and six other *C. difficile* isolates (ribotype 26, serotype A5, A4, A2, K, and D). Figure 5.2(b) shows the Western blot result when the SLPs were tested for their reactivity with antiserum against isolate 683. As anticipated a strong reaction to both of the SLPs extracted from isolate 683 was observed. A cross-reaction to both of the SLPs from isolate 4217 corresponding to ribotype 26 which has an identical SLP profile to that of isolate 683. Cross-reaction to the heavier SLP from the other isolates tested using antiserum to 683 was observed, suggesting antigenic similarities.

Figure 5.3(a) shows the SLP profile of *C. difficile* isolate 604 and six other *C. difficile* isolates (mprl 1123, 2520, 683 and serotypes A8, A9, A3). Figure 5.3(b) shows the Western blot result when the SLPs shown in figure 5.3(a) were tested for a reaction with the antiserum raised to *C. difficile* isolate 604. Cross-reaction of varying intensity to both SLPs from isolates 1123, A8, A9 and A3 was observed. Again cross-reaction with the heavier protein from most isolates tested with antiserum 604 was observed.

Figure 5.2
(a) SDS-PAGE of S-proteins from seven *C. difficile* isolates including laboratory isolate 683.

(b) Western blot of S-proteins from *C. difficile* isolates shown in figure 5.2(a) using antiserum raised against laboratory isolate 683.

Figure 5.2(a)

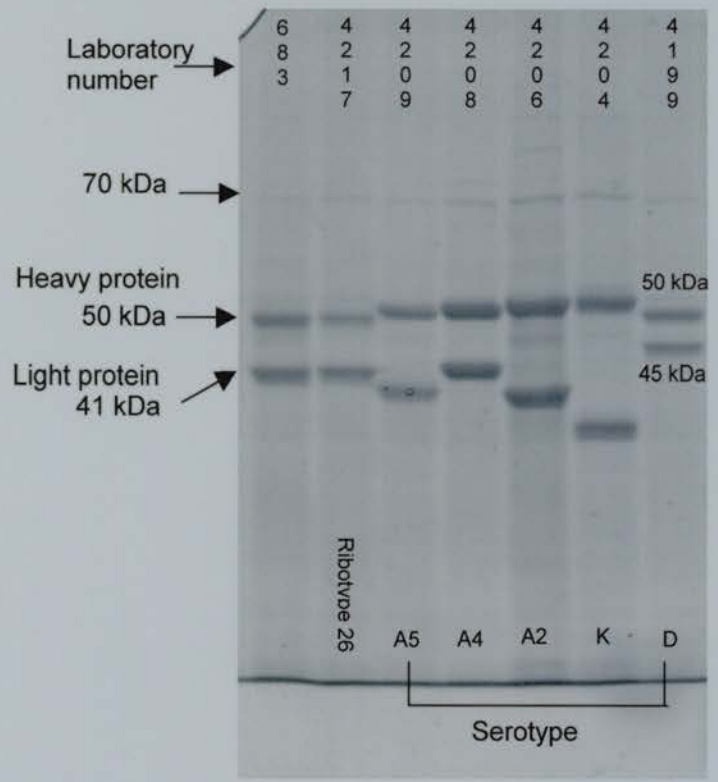


Figure 5.2 (b)

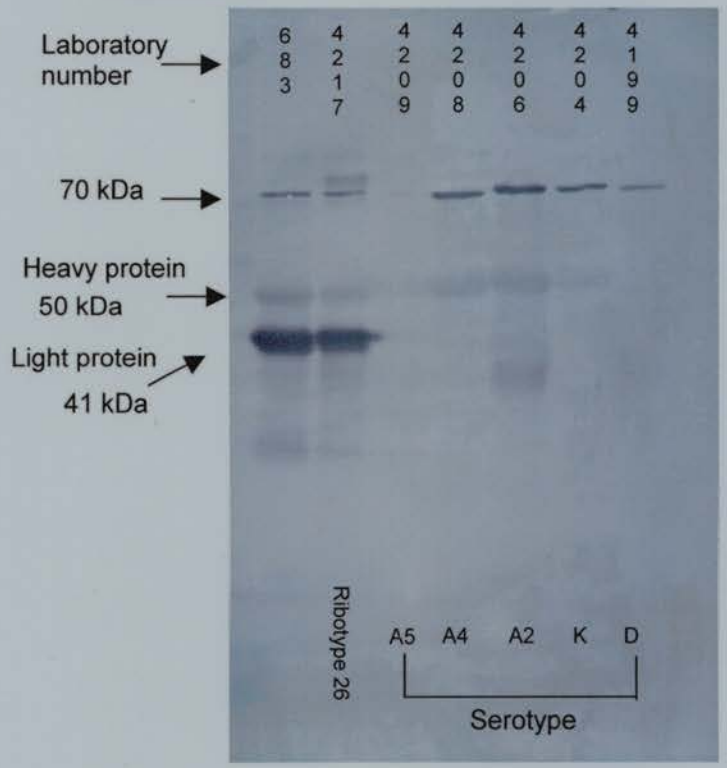


Figure 5.3.
(a) SDS-PAGE of the S-proteins from seven C. *difficile* isolates including laboratory isolate 604.

(b) Western blot of S-proteins from *C. difficile* isolates shown in figure 5.3(a) using antiserum raised against laboratory isolate 604.

Figure 5.3(a)

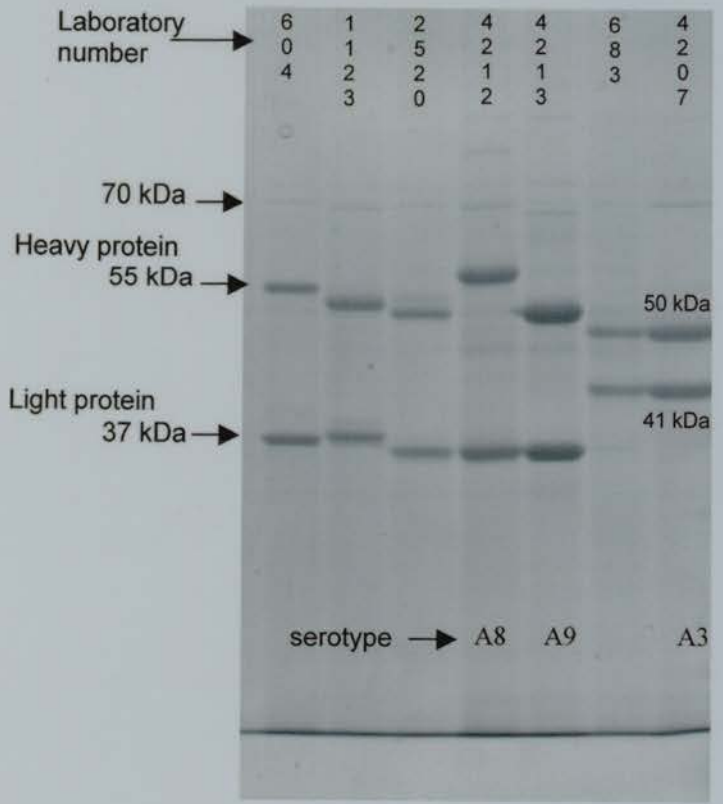


Figure 5.3(b)

Antisera raised to both 2520 and 1128 was tested with the SLPs from six isolates (serotypes C, G, K, I, F, A9). Figure 5.4(a) shows the SLP profile of 1128 and the six other isolates listed above. Figure 5.4(b) shows the resulting Western blot using antiserum to 1128. The antiserum raised against 1128 cross-reacted with both the SLPs from the isolate representing serotype F. There was also a relatively strong cross-reaction observed between the heavier SLP from each of the six isolates tested. The antiserum raised against 2520 (NCTC 11223) cross-reacted with the SLPs from the isolates representing serotype C and K. A strong cross-reaction with the heavy SLP was observed for all of the other six isolates. The protein profile of 2520 and the six isolates tested against antiserum raised to 2520 are shown in figure 5.5(a) and the Western blot result is shown in figure 5.5(b)

The third minor protein of molecular mass 70 kDa which is common to all isolates cross-reacted with all antisera tested, this is shown in Figures 5.2-5.5. It appears to be an antigen common to most if not all strains.

Figure 5.4
(a) SDS-PAGE of the S-proteins from seven *C. difficile* isolates including laboratory strain 1128.

(b) Western blot of the S-proteins from *C. difficile* shown in figure 5.4(a) using antiserum raised against laboratory isolate 1128.

Figure 5.4(a)

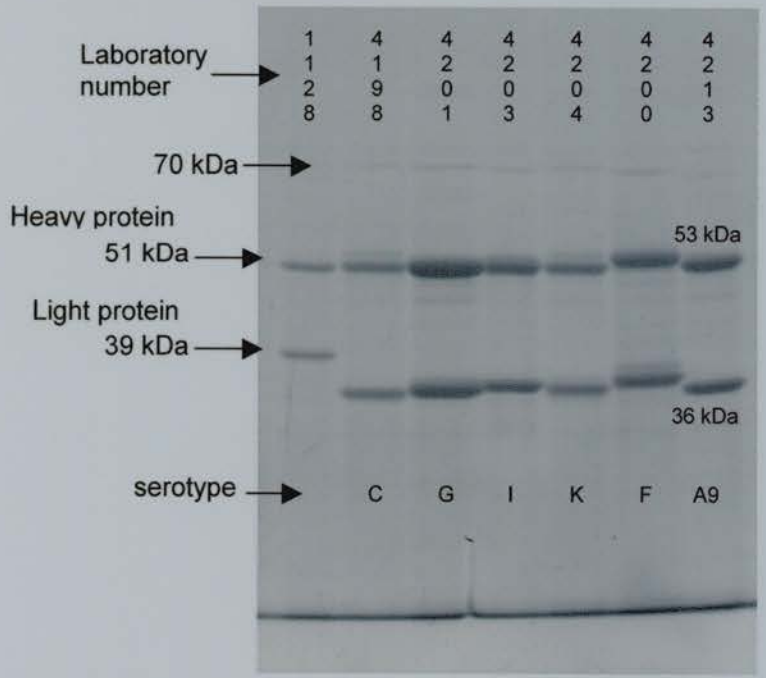


Figure 5.4(b)

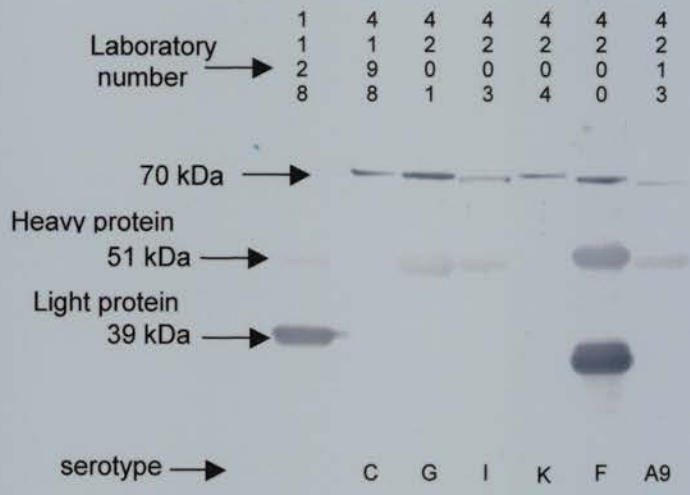
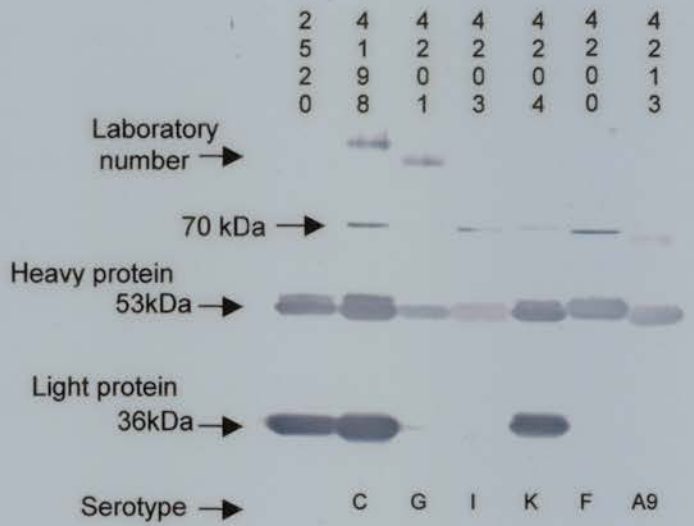


Figure 5.5
(a) SDS-PAGE of the S-proteins from seven *C. difficile* isolates including laboratory isolate, mprl 2520 (NCTC 11223).

(b) Western blot of the S-proteins from *C. difficile* isolates show antiserum raised against laboratory isolate 2520.



5.4. Discussion

This investigation demonstrated that the treatment of whole washed *C. difficile* cells with 5M guanidine hydrochloride extracts two major cell surface proteins which are thought to be the SLPs. A study by Kawata et al (1984), showed that treatment of the outer cell wall extracts with urea or guanidine hydrochloride produced almost identical protein profiles on SDS-PAGE. More recently, Cerquetti et al (2000), demonstrated that complex preparation of cell walls was not required for the extraction of SLPs from *C. difficile*, and that treatment of whole cells with urea led to the extraction of SLPs. We therefore conclude that the two major proteins extracted from *C. difficile* treatment with guanidine hydrochloride are the SLPs.

This study revealed a high degree of variation in the molecular mass of the two SLPs among *C. difficile* isolates of different serotype and ribotype. Other investigators have described similar variation of these proteins (Takeoka et al, 1991; Cerquetti et al, 2000). This variation is not unique to *C. difficile*, *Campylobacter fetus* can express SLPs of different molecular mass (Nitta et al, 1997) and *Lactobacillus acidophilus* can also express different SLPs (Boot et al, 1995). It is generally thought that this variation may be a means of avoiding recognition by the immune system.

Further analysis of the SLPs of *C. difficile* using Western blotting revealed that there was significant cross-reaction of antiserum with the heavier SLPs from different *C. difficile* isolates, suggesting some degree of antigenic similarity. However, there was very little evidence of cross-reaction of antiserum with the lighter SLP from isolates of different serotype or ribotype, suggesting that this lighter protein is antigenically distinct between strains. These data agree with that of Cerquetti et al (2000), and more recently Calabi et al (2001), which states that no antigenic relationship was

observed among SLPs of lower molecular masses and that there was cross-reactivity among the SLPs of high molecular mass from different strains. Calabi et al (2001), also showed that there was significant homology of the amino acid sequence of the heavier SLPs among different strains. However, there was much less homology among the lighter of the two SLPs among different strains.

Western blotting also indicates that the lighter SLP may be more immunogenic than the heavier protein due to the consistently stronger reaction of antiserum with the lighter SLP which is observed in the Western blots. This interpretation is taken with caution, as Western blotting is not quantitative.

The role if any, of the SLPs in virulence has yet to be determined as is the importance of any possible immune response to these proteins. However, the findings from the study described and those of other workers indicate that the SLPs are immunogenic and that there may be an immune response to these proteins in human disease. Pantosti et al (1989), showed that IgG from patients with antibiotic associated diarrhoea recognised the lighter molecular mass SLPs. However, it is the mucosal antibody response in the gut which is important in such infections and this has not been demonstrated. It could be postulated that an IgA response to the SLPs in the gut may offer protection from colonisation or infection with *C. difficile*. The variation described, particularly of the lighter SLP may provide a means of avoidance of the immune system as previous infection or colonisation with *C. difficile* would not offer protection from re-infection with a different strain with distinct SLPs.

The data discussed indicate that further investigation of the SLPs of *C. difficile* is required to determine their role in virulence. However, this variation is also a

phenotypic characteristic which has the potential to be exploited as the basis of a phenotypic typing method. The SDS-PAGE protein profiles of the *C. difficile* SLPs are extremely simple, they are reproducible and can be easily interpreted. Application of this SLP variation in *C. difficile* as the basis of a typing method will be discussed in Chapter six.

CHAPTER SIX

Assessment of the novel phenotypic S-typing method for *C. difficile* and the epidemiology of *C. difficile* S-types in patients and their environment.

AIMS

1. To determine the number of different *C. difficile* S-types that could be identified from the patients and their environment in wards 5 and 6, RVH.
2. To compare S-typing of *C. difficile* with PCR ribotyping.
3. To investigate the epidemiology of *C. difficile* S types from patients and their environment in wards 5 and 6, RVH.

RESULTS

6.1. S-typing of *C. difficile* isolates by analysis of the S-layer protein profiles

The high degree of variation of the molecular masses of the SLPs amongst *C. difficile* isolates of different serotypes and ribotypes has been described in Chapter 5, and as demonstrated, the two SLPs produce a very simple pattern on SDS-PAGE. This variation and the simple protein patterns produced were exploited as the basis for the development of a phenotypic typing method for *C. difficile*. As described in Chapter 5, the SLPs can be extracted by treatment with guanidine hydrochloride and visualised on SDS-PAGE. Each isolate of *C. difficile* can be designated a four-digit S-type (strain) number based on the molecular weights of the two SLPs and the S-type number can be determined using Phoretix™ software to calculate the molecular

masses of the two SLPs. The *C. difficile* isolates collected from the patients and the environment of wards 5 and 6, RVH were subjected to the S-typing method and each isolate was subsequently given an S-type number.

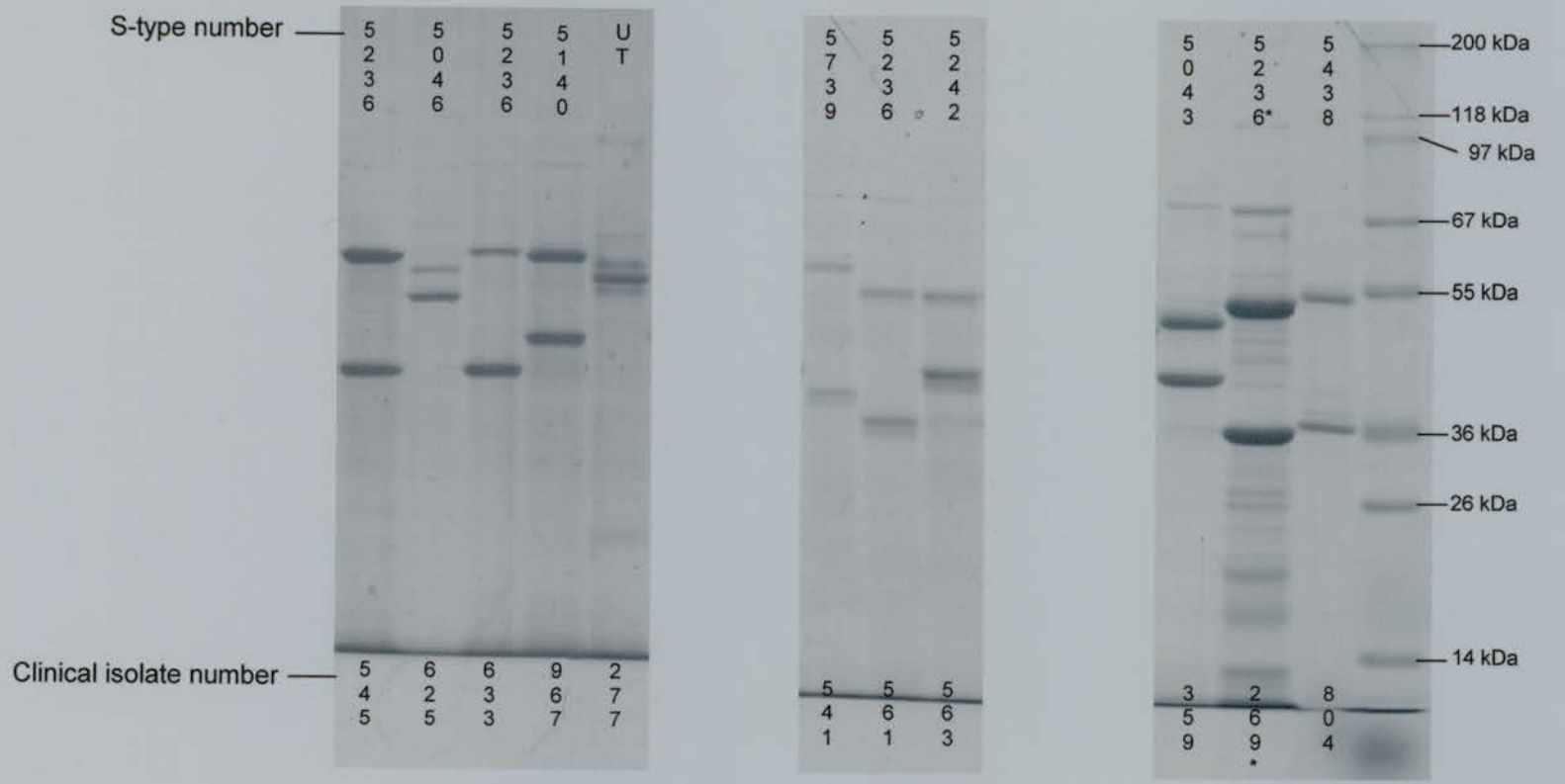
6.1.1. S-typing of *C. difficile* isolates from patients in wards 5 and 6, RVH

A total of 206 isolates from 100 patients in RVH were subjected to S-typing and each isolate was designated an S-type number. A total of eight distinct S-types were identified within the collection of isolates from patients in wards 5 and 6 RVH.

In addition to the SDS-PAGE analysis of the S-layer protein profiles, the *C. difficile* isolates were tested for their ability to produce *C. difficile* toxin(s) by the use of the Techlab™ ELISA test kit for toxins A and/or B. This revealed that within S-type 5236, some isolates produced toxin(s) and other isolates did not. This allowed further differentiation within S-type 5236, and a total of nine different S/toxin-types were identified. The S-layer protein profiles from representative isolates of each of the eight S-types identified are shown in figure 6.1. Details of the S-type, the toxigenic potential and the frequency of isolation are summarised in table 6.1.

Toxigenic S-type 5236 was isolated from 78% patients and accounted for 150 of the 206 isolates from patients in RVH. It was evident from the data that toxin producing S-type, 5236 was the endemic strain of *C. difficile* colonising the patients in wards 5 and 6, RVH. The other S- types were isolated in comparatively small numbers and were associated with very small numbers of patients.

Figure 6.1. SDS-PAGE of the S-layer protein profiles from 11 clinical isolates representing each of the 9 *C. difficile* S/toxin-types isolated from patients in wards 5 and 6, RVH.



* non-toxin producing S-type 5236

Table 6.1. The frequency of isolation and the toxigenic potential of each of the S-types isolated.

S-TYPE	TOXIGENICITY	NUMBER (%) OF ISOLATES	NUMBER AND %[1] OF PATIENTS
5236	toxigenic	150 (73)	78
5236	non-toxigenic	8 (4)	4
5242	toxigenic	27 (13)	16
5739	toxigenic	1(0.5)	1
5438	toxigenic	5 (2)	5
5140	toxigenic	5 (2)	3
5144	non-toxigenic	6 (3)	6
5043	toxigenic	1(0.5)	1
UT[2]	toxigenic	3 (1.5)	1
Total		206	

[1] As there were 100 patients in the study, the number and percentage of patients are equivalent. The total percentage is more than 100, as 14 patients were colonised with more than one *C. difficile* S-type.

UT[2]: this toxigenic strain did not produce two distinct S-proteins on SDS-PAGE, thus an S-type number was not designated.

6.1.2. S-typing of isolates from the environment of wards 5 and 6, RVH

Isolates collected from the environment of wards 5 and 6 were also subjected to S-typing. Isolates were collected from a total of 185 areas within wards 5 and 6, and 203 isolates from 168 of these areas were subjected to S-typing. Toxin tests were not performed on all of the environmental isolates, thus S-type 5236 could not be differentiated into toxin producing and non-toxin producing strains. A small selection of isolates representing all three S-types were tested for toxin production and all

tested positive. As the non-toxin producing S-type 5236 occurred in small numbers in the patient population, it is likely that this would be the only discrepancy and would not affect the overall results to a significant extent.

Only three of the eight S-types (5236, 5242 and 5438) that were isolated from patients were also isolated from the environment, no other S-types were identified. The frequency of isolation of each of these three S-types is shown in table 6.2.

Table 6.2. The S-types isolated and their frequency of isolation from the environment of wards 5 and 6, RVH.

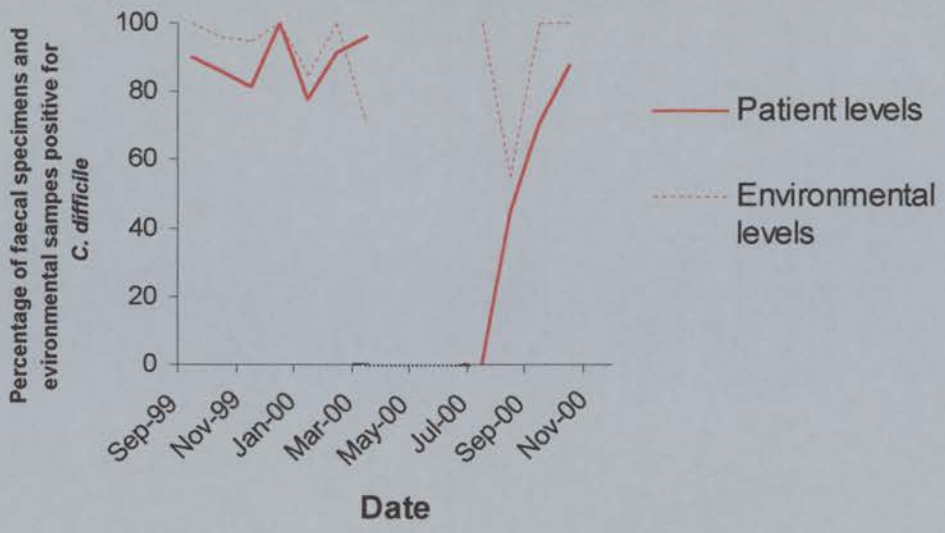
S-TYPE	NUMBER (%) ISOLATES
5236	184 (91)
5242	15 (7)
5438	4 (2)

The data relating to S-types isolated from the environment correlate well with the S-types isolated from the patients in wards 5 and 6, RVH. Toxigenic S-type 5236 was the most frequently isolated strain from patients and correspondingly it was the most frequently isolated strain from the environment of these patients. The other minor S-types were not isolated from the environment, this is likely to reflect their low numbers or absence from the areas of the environment which were sampled.

6.1.3. Temporal association between environmental contamination and patient colonisation with three different S-types of *C. difficile*

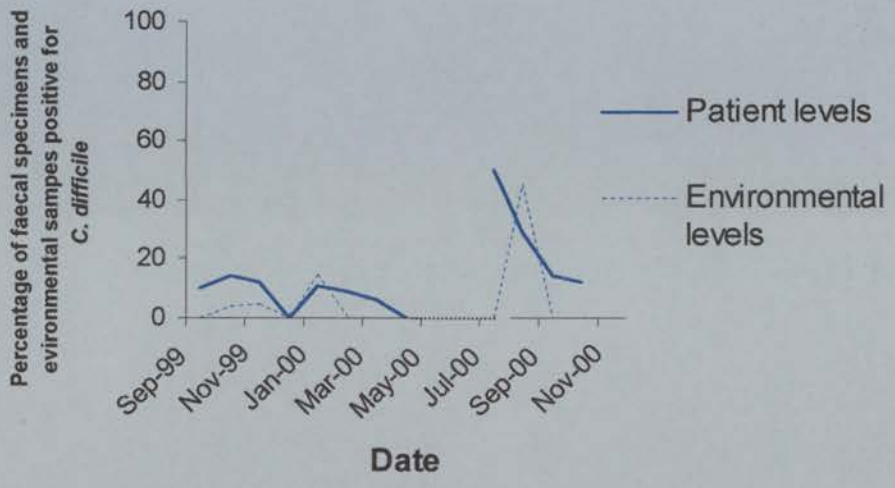
Figure 6.2 shows the level of isolation of *C. difficile* S-type 5236 from patients and the environment of wards 5 and 6, RVH over a 15-month period. The levels of isolation of S-type 5236 from the environment and from patients show similar trends. Figure 6.3 shows the levels of isolation of S-type 5242 from the patients and the environment of ward 5 and 6, RVH, again the trends for both patients and their environment are similar.

Figure 6.2. The level of isolation of S-type 5236 from the patients and the environment of wards 5 and 6, RVH.



* No isolates were collected between 29/04/00 and 17/06/00 due to the illness of the research nurse.

Figure 6.3. The level of isolation of S-type 5242 from the patients and the environment of wards 5 and 6, RVH



* No isolates were collected between 29/04/00 and 17/06/00 due to the illness of the research nurse.

It is of interest that the S-type 5438 was first isolated from the environment on 20/03/00 and followed the initial isolation of this S-type from a patient on 09/03/00. The data in figures 6.2 and 6.3 suggest that environmental contamination may be associated with patient colonisation.

6.2. Comparison of S-typing with PCR ribotyping of *C. difficile*

Eleven *C. difficile* isolates collected from the study of patients in RVH were typed by Dr Jon Brazier, ARU, PHLS, Cardiff, Wales. The eleven isolates represented seven different S/toxin-types and when typed at ARU, seven different PCR ribotypes were identified. The PCR-ribotype patterns produced by these isolates were used as

"standards" to facilitate the typing of other isolates collected during the study in RVH. The S-types of the eleven representative isolates and the results from the PCR ribotyping carried out at ARU, Cardiff are shown in table 6.3.

Table 6.3. The S-types and PCR ribotypes of eleven *C. difficile* isolates. (PCR ribotyping carried out at ARU, Cardiff, Wales.)

S-TYPE (NUMBER TESTED)	PCR RIBOTYPE (NUMBER TESTED)
5236 T (3)	type 1 (3)
5236 NT (2)	type 9 (1) type 10 (1)
5144 NT (1)	type 10 (1)
5242 T (1)	type 14 (1)
5043 T (1)	type 26 (1)
5438 T (1)	type 12 (1)
UT /T(2)	type 5 (2)

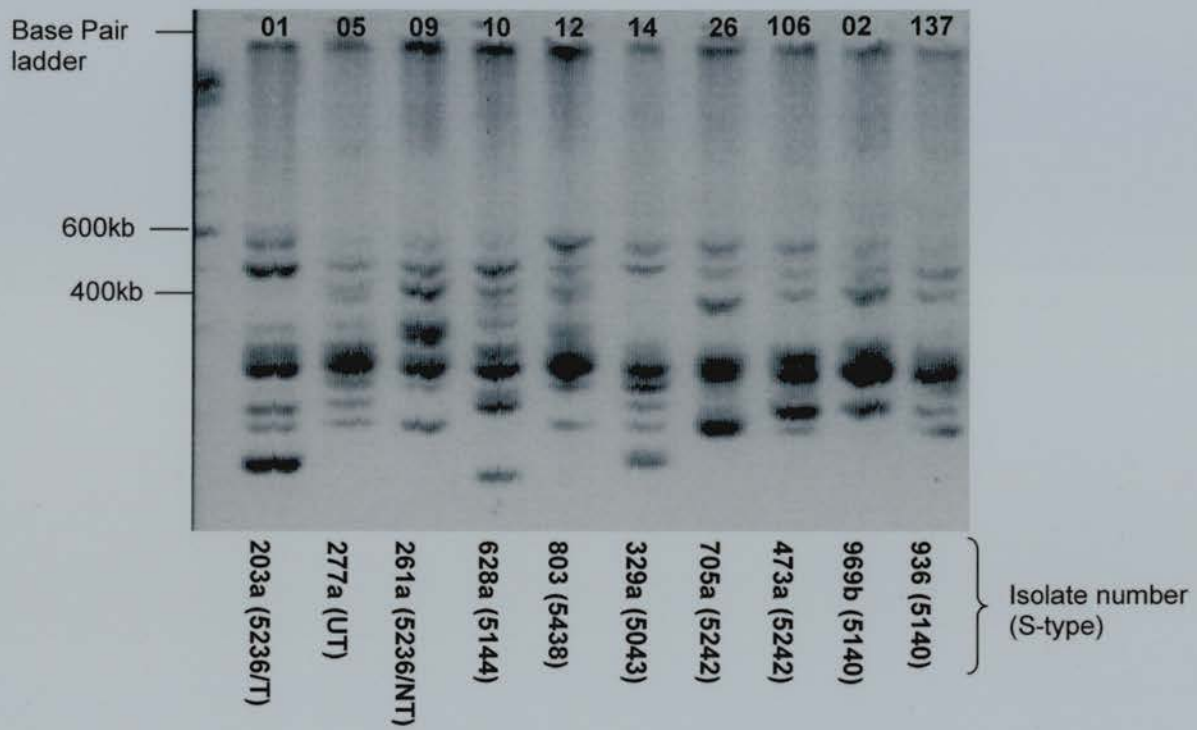
For the purposes of comparing PCR ribotyping and S-typing, a further 37 *C. difficile* isolates which represented eight different S/toxin-types were typed by the PCR ribotyping method. Isolates were chosen to represent the entire study period. The results are shown in table 6.4 and include the results from the eleven isolates typed by Dr Jon Brazier, ARU, Cardiff.

The investigation identified ten PCR ribotypes within the group of eight S/toxin-types tested. A representative of each of the ten PCR ribotypes is shown in figure 6.4.

Table 6.4. The PCR ribotypes and S-protein types of the 48 *C. difficile* clinical isolates collected from patients in RVH

S-protein type (number tested)	PCR ribotype
5336/T (17)	17 type 01
5242/T (12)	8 type 14 4 type 106
5438/T (3)	2 type 12 1 type 14
5043/T	type 26
5144/NT (3)	3 type 10
5336/NT (5)	3 type 09 2 type 10
5140/T (5)	4 type 02 1 ribotype 137
UT/T (2)	2 type 05

Figure 6.4. Summary of the 10 PCR ribotypes identified from a selection of *C. difficile* isolates collected from patients in RVH.
The isolate number and the S-type are given on the figure.



There were two ribotypes identified within four of the S-types (5242, 5140, 5236/NT and 5438) and within ribotypes 10 and 14, there was more than one S-type. The data indicates that PCR ribotyping offers more discrimination than S-typing, but that within at least ribotypes 10 and 14 there is more than one phenotype. The results also show that the toxigenic S-type 5236 correlates to PCR ribotype 1 for all isolates tested, and it is likely that all isolates identified as toxigenic S-type 5236 were in fact PCR ribotype 1.

6.3. Discussion

6.3.1. Assessment of S-typing and comparison to PCR ribotyping.

Historically, the typing methods used for *C. difficile* were based on the phenotypic features of the organism. Techniques such as bacteriophage and bacteriocin methods (Sell et al, 1983), serotyping (Delmee et al, 1985), radio-PAGE (Tabaqchali et al, 1984b) and immunochemical fingerprinting methods (Poxton et al, 1984) have all been used to type *C. difficile*. Phenotypic methods have been recently replaced by more sophisticated genotypic techniques. Several genotypic methods have been developed, such as AP-PCR (McMillin & Muldrow, 1992) and PFGE which is highly discriminatory, but is complex and labour intensive (van Dijck et al, 1996). In addition, problems have been reported with DNA and typeability by PFGE (Kato et al, 1994). Hyett et al (1997), found that many of the untypeable isolates belonged to PCR ribotype 1 and suffered from DNA degradation. PCR ribotyping was initially used by Gurtler (1993), and the method was adapted and improved by Cartwright et al (1995) and O'Neill et al (1996). PCR ribotyping has proved to be the best of the genotypic methods, and is currently the typing method of choice in the ARU, Cardiff, and therefore it was considered the most useful method for this comparative study.

The novel S-typing method assessed in this study showed a good level of discrimination, a total of eight S-types were easily identified and visualised on SDS-PAGE. These eight types were further discriminated to nine types by testing isolates for their ability to produce toxin(s). As both non-toxigenic and toxigenic strains occurred within one S-type toxin testing would be required to complement S-typing.

The techniques involved were simple and the protein profiles were very easy to interpret. The SLPs have been shown to be stable within any one isolate and the results are highly reproducible (Sharp and Poxton, 1985). Some difficulties did occur when S-types with very similar, however different protein profiles. Current gel analysis software packages, such as Phoretix™ can be used to assist in molecular weight calculation and differentiation of very similar S-types.

Comparison of S-typing with PCR ribotyping determined that PCR ribotyping offered slightly more discrimination, in total ten PCR ribotypes were identified compared to only nine S/toxin-types. The findings from this investigation showed that there was more than one ribotype within a phenotype and vice versa. It was not surprising that PCR ribotyping could further differentiate S-types, it is however of interest that different phenotypes could occur within a genotype. As it is the phenotypic characteristics of an organism which interact with the host to cause, or exacerbate the pathological effects of the disease, the use of phenotypic typing methods for epidemiological purposes should not be under-rated.

There was excellent correlation between the endemic toxigenic S-type 5236 and PCR ribotype 1, the results indicate that PCR ribotype 1 was responsible for the colonisation of 78% of the *C. difficile* culture positive patients in this study. This is not surprising and correlates well to the data from the PHLS for England and Wales which describes PCR ribotype 1 as the endemic strain in 57% hospitals in England and Wales (Brazier et al, 1997b).

6.3.2. The epidemiology of *C. difficile* S-types from patients and their environment.

S-typing and toxin testing of isolates from patients and the environment of wards 5 and 6, RVH identified a total of nine distinct S/toxin-types from the 206 patient and 203 environmental isolates tested during the 17-month study period. As discussed the endemic strain, toxigenic S-type 5236 which corresponded to PCR ribotype 1 colonised three-quarters of all the patients from whom *C. difficile* was isolated. The eight other S/toxin -types colonised a comparatively small number of patients. These findings were similar to those from a study carried out by Fawley and Wilcox (2001). Their study investigated the patients and the environment of two elderly general medicine wards over a 22-month period. Typing by RAPD and ribospacer polymerase chain reaction (RS-PCR) identified eight distinct genotypes from 201 *C. difficile* isolates from patients and their environment. Their study identified four different genotypes from the patients investigated, and one of these genotypes, corresponded to PCR ribotype 1 and accounted for 92% of all the patient isolates (Fawley and Wilcox, 2001).

The toxigenic S-type 5236 which was endemic in patients in RVH also accounted for the majority of the RVH environmental isolates. Two of the other eight S/toxin-types that were isolated from patients were also isolated from the environment, suggesting a possible association between the S-types isolated from patients and their environment.

Other studies have made associations between the contamination of the environment with specific strains and colonisation of patients with the same strains. Cohen (2000), showed that the AP-PCR types identified from patients in a geriatric and general medicine unit correlated closely with the types identified from the environment of

these patients. Also, Delmée et al (1988), identified a relationship between the colonisation of neonates with particular serogroups of *C. difficile*, clusters of carriage with a particular serogroup and contamination of the patient environment with the same serogroups. However, a study in an oncology ward identified 18 different AP-PCR types from patients and a further nine different AP-PCR types from the environment. There was no correlation between the types isolated from the environmental and the types isolated from patients in the oncology unit. This suggested that in this endemic situation, cross-contamination from the ward environment may not have been the source of colonisation, but that *C. difficile* infections were the result of an endogenous source (Cohen et al, 1997).

Further analysis of the levels of isolation of individual S-types from the environment and patients in RVH indicated an association between the levels of certain *C. difficile* S-types isolated from patients and the levels of these S-types isolated from the environment. There was evidence of peaks of environmental contamination with S-types 5236 and 5242, corresponding with peaks in the levels of 5236 and 5242 isolated from patients. The data showed that peaks of contamination in the environment with a particular strain may occur just before or just after a rise in the number of patients colonised with the same strain. Interestingly, S-type 5438 was isolated from two patients in ward 5 and then several months later from three patients in ward 6. All the patients colonised with this strain were housed in different bays suggesting that contamination of the environment may not have been directly responsible if cross-infection occurred with this strain. In the study by Fawley and Wilcox (2001), they reported that a particular genotype was not isolated from the environment until the sixth patient in a cluster of cases became symptomatic. This

data from both RVH and Fawley and Wilcox (2001), suggests that an increase in the number of colonised patients can lead to an increase in the levels of environmental contamination with the colonising strain. Fawley and Wilcox (2001), also suggested that initial cross-infection from patient to patient or from staff to patient may occur before heavy environmental contamination occurs and causes further cross-contamination. This may perhaps explain the transfer of S-type 5438 between patients in different bays within ward 5. However, other sources such as commodes and equipment which is shared between bays within wards may be the common source of infection.

Due to the endemic nature S-type 5236 in both the environment and the patients, it was not possible to determine if environmental contamination or patient to patient and staff to patient spread was the main source of cross-infection in wards 5 and 6, RVH. Fawley and Wilcox (2001), encountered similar difficulties in determining the source of infection. They identified six different genotypes from the environment. However, only two of these were also isolated from patients and the endemic genotype corresponding to PCR ribotype 1 accounted for 92% of the environmental *C. difficile* isolates.

In summary, S-typing is a simple typing method which appears to offer good discrimination and reproducible results. From the isolates tested in this study S-typing showed good correlation with PCR ribotyping, although typing of a diverse range of isolates would be required to determine the real correlation with PCR ribotyping. S-typing demonstrated the endemic nature of one S- type amongst a total of nine different types isolated from patients and the environment of wards 5 and 6,

RVH. This endemic S-type corresponded to PCR ribotype 1 which is the endemic strain in the rest of the UK.

This study and others have demonstrated substantial evidence for environmental contamination with *C. difficile*, the *C. difficile* types which are isolated from the environment reflect those types which colonised *C. difficile* culture positive patients. Much epidemiological evidence for the role of environmental contamination in the transfer of infection has been demonstrated, however the relative importance of environmental contamination, patient to patient and staff to patient cross-contamination have to be determined. This stresses the paramount importance of good hand washing procedures by all health workers and the need for good ward cleansing practices at all times in order to reduce the opportunity for cross-contamination.

CHAPTER SEVEN

Colonisation with different S-types of *C. difficile* and the development and severity of diarrhoea.

AIMS

1. To determine if colonisation with different S-types of *C. difficile* had any effect on the development of, and the duration and severity of patient symptoms.
2. To determine if colonisation with more than one S-type of *C. difficile* was associated with the level of symptomatic colonisation and/or the duration and number of episodes of diarrhoea.

Each *C. difficile* isolate collected from patients in ward 5 and 6, RVH was designated an S-type number as described in Chapter 6. One isolate from each of the 206 positive stool specimens was typed. More than one specimen was collected from most patients and more than one S-type was isolated from some patients. The data relating to patient symptoms; the duration of diarrhoea and the number of episodes of diarrhoea was investigated to determine if there was any association between S-type and the development of symptoms and the severity of the disease.

RESULTS

7.1. Symptoms in patients colonised with different *C. difficile* S-types

Of the 100 patients from whom *C. difficile* was cultured, 46% were symptomatic, that is they had at least one episode of diarrhoea within the period of two weeks prior

to, and/or two weeks after a culture positive stool specimen was identified. Some of the patients may have been previously asymptomatically colonised.

Of the 100 patients from whom *C. difficile* was detected by culture, 86 patients were colonised by one S-type only and the remaining 14 patients had more than one S-type of *C. difficile* isolated from several consecutive faecal specimens.

7.1.1. Diarrhoea in patients colonised with different *C. difficile* S-types

Each of the nine *C. difficile* S/toxin-types isolated from patients in RVH and the number of symptomatic patients who were colonised with each of these S-types are shown in table 7.1. Toxigenic S-type 5236 was clearly the most frequently isolated S-type, it was isolated from a total of 78% of *C. difficile* culture positive patients investigated during the study, and it was associated with symptomatic colonisation in just over 51% of these patients. Of the patients colonised with the second most frequently isolated S-type, 5242, 38% were symptomatically colonised. However, as a number of patients were colonised by more than one S-type, it would be misleading to take the data in Table 7.1, at face value, as colonisation with more than one S-type may affect the likelihood of a patient developing symptoms. In addition, the number of patients colonised with each of the S-types, apart from toxigenic type 5236 was very small and thus the data could not be reliably statistically analysed to determine the significance in the levels of symptomatic colonisation by different S-types of *C. difficile*.

Table 7.1. The number of symptomatic and asymptomatic patients who are colonised with each of the *C. difficile* S-types isolated from patients in wards 5 and 6, RVH.

S-TYPE	NUMBER (%) OF PATIENTS		
	SYMPTOMATIC	ASYMPTOMATIC	TOTAL*
5236 T	40 (51)	38 (49)	78
5236 NT	2 (50)	2 (50)	4
5242 T	7 (44)	9 (56)	16
5739 T	0 (0)	1 (100)	1
5438 T	2 (40)	3 (60)	5
5140 T	1 (33)	2 (67)	3
5144 NT	4 (67)	2 (33)	6
5043 T	1 (100)	0 (0)	1
UT/T	1 (100)	0 (0)	1

*The total number of patients is more than 100% because more than one S-type was isolated from some patients.

T= toxin producing; NT=non-toxin producing
UT=untypeable

It is generally thought that non-toxigenic strains of *C. difficile* do not cause symptoms in colonised patients. It is interesting therefore to note that of the ten patients who were colonised with only non-toxigenic S-types, six patients had diarrhoea. The reason for their symptoms is unconfirmed, and may not be related to *C. difficile.*

Of the 26 patients who met the criteria for CDAD, 24/26 were colonised with toxigenic S-type 5236, and four of the patients were colonised with two S-types

including toxigenic S-type 5236. One of the remaining two patients was colonised with 5242 and the other patient with both 5438 and 5140. This implicates toxigenic S-type 5236 in 92% of cases of CDAD and it is likely to be the sole cause in 85% of CDAD cases.

7.2. Symptoms in patients colonised with only one S-type of *C. difficile*

7.2.1. Diarrhoea in patients colonised with only one S-type of *C. difficile*

A total of 86 patients were colonised by one S-type only, the colonising S-type and the number of symptomatic patients are shown in table 7.2.

Forty-six percent of patients who were colonised with toxigenic S-type 5236 only had diarrhoea compared to only 24% of patients colonised by any one of the other S-types. The level of symptomatic colonisation was compared for patients colonised with toxigenic S-type 5236 only and for patients colonised with any one of the other S-types identified in the study.

The data was analysed by the Chi^2 test and the statistical analysis showed that the higher level of symptomatic colonisation in those patients colonised with S-type 5236 compared to those who were colonised with any one of the other S-types was insignificant (Mann Whitney U test, $p=0.06$). However, this is a borderline insignificant result, and hence colonisation with toxigenic S-type 5236 may be associated with an increased level of symptomatic colonisation when compared to the other S-types. Further investigation is required to determine the significance, if any of this result.

Table 7.2. Diarrhoea in patients colonised by only one toxigenic S-type of *C. difficile*.

	NUMBER (%) OF PATIENTS		
S-TYPE	SYMPTOMATIC	ASYMPTOMATIC	TOTAL
5236 T	30 (46)	35 (54)	65
5242 T	3 (27)	8 (73)	11
5739 T	0 (0)	3 (100)	3
5941 T	0 (0)	1 (100)	1
5144 T	2 (50)	2 (50)	4
5140 T	0 (0)	2 (100)	2
TOTAL	35 (41)	51 (59)	86

ıer" S-types 24%
of a total of 21)
mptomatic patients

T= toxin producing

7.2.2. The number of episodes and the duration of diarrhoea in patients colonised with only one S-type of *C. difficile*

The number of episodes and duration of diarrhoea were compared in patients colonised with toxigenic S-type 5236 only, and in patients colonised by any one of the other S-types of *C. difficile*. The range and mean duration of symptoms and number of episodes of diarrhoea in these two patient groups is given in table 7.3.

Table 7.3. The duration of symptoms and the number of episodes of diarrhoea in patients colonised with toxigenic S-type 5236 and in patients colonised with any one of the other S-types.

S-TYPE (NUMBER OF PATIENTS)	DURATION IN DAYS OF SYMPTOMS		EPISODES OF DIARRHOEA	
	RANGE	MEAN	RANGE	MEAN
5236 (n=30)	1-49	8	1-12	3
"Other" (n=5)	1-7	3	1-4	2

Statistical analysis using the Mann Whitney U test determined that there was no significant difference in the number of episodes of diarrhoea (p=0.59), or the duration of symptoms (p=0.09) in patients colonised with toxigenic S-type 5236 compared to those patients colonised with any one of the other S-types.

7.3. Symptoms in patients colonised with more than one S-type of *C. difficile*

7.3.1. Diarrhoea in patients colonised with more than one S-type of *C. difficile*

S-typing of *C. difficile* isolates determined that a total of 14 patients were colonised with more than one S-type of *C. difficile,* 13 of these patients were colonised with two S-types and one patient was colonised with three S-types of *C. difficile.* The symptomatic status of the patients colonised by more than one S-type is shown in Table 7.4.

Table 7.4. Diarrhoea in patients colonised with more than one S-type of *C. difficile.*

	S-TYPES			NUMBER OF PATIENTS		
				SYMPTOMATIC	ASYMPTOMATIC	TOTAL
TOXIGENIC S-TYPES ONLY	**5236 T**	**5242 T**		3	1	4
	5236 T	***UT/T***		1	0	1
	5236 T	**5043 T**		1	0	1
	5236 T	**5438 T**	**5242**	1	0	1
	5140 T	**5438 T**		1	0	1
TOXIGENIC AND NON-TOXIGENIC S-TYPES	**5236T**	**5144 NT**		2	0	2
	5236 T	**5242 T**		2	2	4
	Total			11	3	14

T= toxin producing; NT=non-toxin producing
UT=untypeable

Of those patients colonised by more than one S-type, 79%(11/14) were symptomatic compared to only 41% (35/86) of those patients who were colonised by only one S-type. When the level of symptomatic colonisation was compared between the two patient groups using the Chi^2 test, the result ($p= 0.01$) indicated that colonisation with more than one *C. difficile* S-type(s) is significantly associated with symptomatic colonisation with *C. difficile*.

7.3.2. The number of episodes and the duration of diarrhoea in patients colonised more than one S-type of *C. difficile*

The number of episodes and the duration of diarrhoea were compared in symptomatic patients colonised with one S-type only and in those patients colonised with more than one S-type of *C. difficile*. The mean and range of the duration of symptoms and number of episodes of diarrhoea is shown in Table 7.5.

Table 7.5. The duration and the number of episodes of diarrhoea in patients colonised with only one S-type and in those patients colonised with more than one S-type of *C. difficile*.

	DURATION OF SYMPTOMS IN DAYS		NUMBER OF EPISODES	
S-TYPES (n= number of patients)	**RANGE**	**MEAN**	**RANGE**	**MEAN**
One S-type only (n=35)	1-49	7	1-12	3
More than one S-type (n=10)	1-10	5	1-8	3

Statistical analysis of the data summarised in table 7.5, using the Mann Whitney U test determined that there was no significant difference in the duration of symptoms ($p=0.58$), or the number of episodes of diarrhoea ($p= 0.58$) in those patients who were colonised with only one type of *C. difficile* and those patients who were colonised with more than one S-type of *C. difficile*.

In conclusion, toxigenic S-type 5236 was isolated from 78% of culture positive patients in wards 5 and 6, RVH. It was associated with 92% of cases of CDAD. This study did not provide any evidence that a particular S-type may be associated with

symptomatic colonisation, or an increase in the severity of symptoms. Colonisation with more than one S-type is associated with symptoms when compared to colonisation with only one S-type, but is not significantly associated with the number of episodes of diarrhoea or the duration of symptoms.

7.4. DISCUSSION

The aims of this investigation were to determine which S-types were associated with asymptomatic colonisation, which were associated with infection and disease and if the infecting S-type of *C. difficile* affected the severity of the symptoms produced. The effect of colonisation with more than one S-type was also investigated. It was hypothesised that the SLPs might be circumstantially related to the virulence of a particular S-type and therefore the SLPs might be related to the severity of symptoms.

From the study of *C. difficile* isolates collected from patients in RVH it was not possible to draw any conclusions with respect to which S-types were associated with symptomatic or asymptomatic colonisation and which S-types produced the most severe symptoms. This was due to the endemic nature of toxigenic S-type 5236 which accounted for 78% of all *C. difficile* isolates collected from patients in RVH. All of the other S-types were isolated in small numbers and therefore reliable statistical analysis to compare the levels of symptomatic colonisation and severity of symptoms in patients colonised with different S-types was not possible.

Studies have implicated that there is variation in the virulence potential amongst different strains of *C. difficile*. Several epidemiological studies have shown that certain serotypes of *C. difficile* are associated with AAD (serotypes A, C and D). Others are associated with adult asymptomatic carriage (serotype F) and some serotypes with infant colonisation such as serotypes B and F (Delmée et al, 1985 and Delmée et al, 1988).

Data from the International Typing Study on *C. difficile* has implicated certain types, such as serotype C and G with hospital outbreaks. Serotype G correlates to PCR

ribotype 1, which has caused most outbreaks in the UK and is the most common type referred to the ARU for England and Wales (Brazier et al, 1997b). As discussed in Chapter 6 toxigenic 5236 correlates to PCR ribotype 1, thus the data from this study correlates well with the findings from the rest of the UK.

In RVH, the endemic S-type 5236, was associated with a symptomatic colonisation rate of 51%, and 92% of patients on the unit who developed CDAD were colonised with S-type 5236. The reason that toxigenic 5236 S-type (PCR ribotype1) has become the most commonly isolated type and the most common cause of *C. difficile* associated infection in the UK is not known.

The strain appears to successfully colonise large numbers of geriatric patients asymptomatically, but also causes a considerable proportion of CDAD. Factors influencing the endemic nature of this strain type in the UK, could be that it sporulates very effectively, or that it associates efficiently with the gut mucosa.

Numerous studies have investigated aspects of the variation of virulence expressed between strains of *C. difficile*. As the two major toxins A and B cause the principal pathology of the disease, the potential for toxin production is the most likely indicator of virulence. Wren et al (1987), demonstrated variation in the amount of toxin-produced between strains of *C. difficile* and showed an association between symptoms of antibiotic associated diarrhoea and the ability of a particular strain of *C. difficile* to produce toxins. However, more recently a study investigated the effect of different strains of *C. difficile* in the hamster model and demonstrated no variation in the ability to colonise and cause death between standard (toxin A + B producers) toxigenic strains of *C. difficile*. However, the colonisation and mortality rate reported

for the toxin A negative, toxin B positive strains of *C. difficile* was significantly less that those toxin A and B producers (Sambol et al, 2001).

These studies indicate that the ability of different strains to produce toxin(s) is an important virulence determinant for *C. difficile* disease. However, it is likely that other virulence factors are involved in disease and that they are important in determining the overall virulence potential of *C. difficile* strains. Until recently it was generally accepted that non-toxigenic strains of *C. difficile* did not cause disease. However, numerous toxin variant strains of *C. difficile* have recently been identified (Rupnik et al, 1998). All of these variant strains posses toxin genes, however deletions in one or both the genes renders them non-functional, thus toxins may not be produced. Some of these toxin variant strains, including non-toxin producing variant stains have been shown to produce an actin-specific ADP-ribosylating transferase (binary toxin) (Stubbs et al,2000). This binary toxin is similar to the *C. perfringens* iota toxin and the *C. botulinum* C2 toxin and may be an additional virulence factor in some *C. difficile* strains (Perelle et al, 1997). This may be an important factor for those symptomatic patients who are colonised by non-toxigenic variant strains of *C. difficile*.

From the study described in Chapter 6.2, which compared S-typing with PCR ribotying, the non-toxigenic strains identified from patients in RVH, are likely to be PCR ribotypes 9,10 and 26. These strains are non-toxigenic, but are not variant toxinotypes, that is, they do not possess the pathogenicity locus which encodes the genes for toxins A and B (Stubbs et al, 2000). To date it has not been determined if such non-toxigenic strains have the ability to produce the binary toxin.

Other potential virulence factors include capsules, hydrolytic enzymes, adhesive factors and the putative SLPs. Seddon et al (1990), related the production of hydrolytic enzymes such as hyaluronidase and collagenase to the relative virulence of certain strains of *C. difficile*. These enzymes are likely to compromise the gut integrity, thus contributing to the pathology of disease and potentially revealing receptors for toxins and further adhesion of the organism. Capsules are well documented as anti-phagocytic factors and have been reported on certain *C. difficile* strains, although Davies and Borriello, (1990) reported the presence of capsules *in vitro*, but they could not determine any association with virulence.

It has been suggested that the SLPs may be involved in the virulence of *C. difficile*. They may have a role in the association and adhesion of *C. difficile* with the gut wall, or alternatively they may play a role in the evasion of the host immune system. As there is a high degree of variation in the SLP profile from strain to strain, it could be postulated that the S-layers produced by certain strains may be more effective adhesion factors or evaders of the host immune system, thus affecting the general virulence of a particular strain.

The study of isolates from patients in RVH demonstrated that different S-types can be isolated from the same patient over time. From the data collected it was not possible to determine if the patients from whom more than one S-type was isolated were colonised concurrently by more than one S-type, or if they were colonised by different S-types at different times. However, consecutive culture results from individual patients indicate that different S-types were often isolated alternatively, this suggests that most of these patients did harbour more than one S-type of *C. difficile* and that they were likely to be colonised with more than one S-type at the

same time. Likewise, as only one isolate from each culture positive stool specimen was S-typed, it is not possible to definitively state that any patient was colonised by one S-type only. Sharp and Poxton (1985), demonstrated that patients could carry more than one S-type of *C. difficile* at the same time and it has also been reported that relapses are often caused by a different strain, which may be of endogenous source, again suggesting colonisation with more than one S-type.

Our study indicated that patients colonised by more than one S-type of *C. difficile* were significantly more likely to develop symptoms than if they were only colonised by a single S-type, however their symptoms did not last longer, nor did they have more episodes of diarrhoea.

In conclusion, toxigenic S-type 5236 might be associated with a greater level of symptomatic colonisation than the other S-types identified in the study and it was associated with the majority of CDAD on wards 5 and 6, RVH. Colonisation with more than one S-type was significantly associated with symptomatic colonisation but was not associated with the severity of diarrhoea.

CONCLUSIONS

C. difficile is a well established nosocomial pathogen, responsible for a significant number cases of antibiotic-associated diarrhoea. The disease is predominately associated with elderly patients who have undergone antibiotic treatment.

The study described in this thesis emphasises the high level of colonisation with *C. difficile* in geriatric patients. *C. difficile* was isolated from 30% of all patients sampled in wards 5 and 6, RVH; 27% of patients in ward 5 and 34% of patients in ward 6 tested positive for the organism by culture and/or toxin detection methods. It was interesting to note that ward 5 had a larger patient turnover, and housed up to six "respite" patients at any one time. The isolation rate at any one time varied between 0 and 67% of patients sampled during the study. Other studies have also shown the level of CDAD and colonisation with *C. difficile* to vary over time (Bender et al, 1986; Gerding et al, 1986). Considerable variation in the levels of colonisation and infection has been observed between different wards and hospitals, and may reflect different patient populations, antibiotic policies and infection control policies.

From the patients investigated, 4% from ward 5 and 11% from ward 6 fulfilled the criteria for the diagnosis of CDAD. The findings from patients investigated in RVH correlate with other studies (McFarland et al, 1989; Gerding et al, 1986;Bender et al, 1986).

However, attention is drawn to the bias on sample collection from patients in RVH. Not all of the admissions to wards 5 and 6 were sampled, and it is likely that specimens were easier to collect from the most debilitated patients and those patients with diarrhoea. Patients with diarrhoea are more likely to harbour *C. difficile* and this study may have overestimated the incidence of *C. difficile* in the geriatric unit.

Environmental contamination with *C. difficile* is well documented, however the role of environmental contamination in the epidemiology of *C. difficile* colonisation and infection is not well defined. Various studies have made an association between the level of environmental contamination, asymptomatic colonisation and CDAD (McFarland et al, 1989; Fekety et al, 1981). *C. difficile* was isolated from 7% and 20% of environmental samples from wards 5 and 6 respectively, and this is likely to reflect the higher rate of symptomatic colonisation in ward 6, which may lead to increased contamination of the environment. Contamination of side-rooms was associated with the presence of colonised patients. Floors, toilet areas and sluice rooms were the most frequently contaminated areas, reflecting the frequency with which these areas are exposed to contamination with faecal material.

Evidence for environmental contamination by both colonised and infected patients is convincing, however the role of the environment as a reservoir in the transmission of the organism is not clear. It is apparent from the findings from RVH and from other studies that good cleaning practices, infection control and antibiotic polices need to be implemented and observed by all staff to reduce both patient colonisation and environmental contamination. Research into the development of effective sporicides against *C. difficile* is essential for efficient decontamination of the hospital environment.

The association of CDAD with increasing age and antibiotic use is well determined. A large number of other risk factors related to underlying disease, medication and medical procedures have been implicated in the disease. Different studies have produced conflicting studies, and the most influential risk factors have been difficult to determine. As well as influencing factors related to medication and medical

procedures, other influences such as environmental contamination and the effects of the immune status may be of paramount influence, but they are difficult to investigate; thus much of the evidence surrounding these factors is circumstantial.

In this investigation it was hypothesised that *C. difficile* disease occurs as a two-step disease process. The proposed two-step model (Starr et al, 1997), suggests that patients undergo the transition from a *C. difficile* culture negative state to an asymptomatic *C. difficile* culture positive state, with no detectable toxin in the faeces. The patient may remain in this asymptomatic state, or the patient may undergo the transition from *C. difficile* culture positive to *C. difficile* culture and toxin positive.

The aim of this study was to determine which of the risk factors investigated significantly influenced both the overall disease process, and each of the two-steps in the proposed model. It was anticipated that the study would provide useful information regarding the risk factors which allow colonisation with *C. difficile,* and the factors that allow or enhance toxin production in high levels in the gut.

The data from the study of patients in RVH indicates that different risk factors significantly influence each of the two steps in the model. The model showed that any antibiotic use, but especially the third generation cephalosporin, ceftriaxone significantly influenced the first step, and other cephalosporins and amoxycillin influenced the second step. This raises questions regarding the mechanisms by which the *C. difficile* colonises and subsequently proliferates and produces large amounts of toxin in the gut.

Further studies are required to determine the most significant factors in each step. Such information may be useful in the development of control strategies to prevent outbreaks of infection. Investigation of the effects of significant risk factors such as

antibiotics on the organism *in vitro* and *in vivo* may help to elucidate the mechanisms of pathogenesis, which are not fully understood.

Clinical isolates collected from patients in RVH were typed using a novel phenotypic typing method. This S-typing method exploited the high degree of variation in the molecular masses of the two S-layer proteins from *C. difficile*. Investigation of the S-layer proteins from *C. difficile* revealed that there was considerable variation in the molecular masses of the two S-layer proteins from different serotypes and ribotypes of *C. difficile*. The proteins were visualised on SDS-PAGE and the patterns were extremely simple and easy to interpret. Calculation of the molecular masses was performed on Phoretix™ software, and showed that in the isolates tested, the protein of heavier molecular weight mass varied between 50-57 kilo Daltons (kDa) and the smaller protein from 35-48 kDa. Each of the clinical isolates was designated a four-digit S-type number based on the molecular masses of the two S-layer proteins.

S-typing of 206 clinical isolates from 100 patients revealed eight different S-types, one of which was further differentiated into two types by testing for the potential to produce toxin(s) *in vitro*. When the novel S-typing method was compared to PCR ribotyping, ten different PCR ribotypes were determined. PCR ribotyping offered better discrimination, but the two methods correlated well. It is important to note that different S-layer phenotypes occurred within a genotype, and *vice versa*, as the ideal genotypic typing method should reflect the phenotypic similarities and variations of the organism, as well as offering good discrimination and reproducibility.

S-type 5236/T was isolated from 78% of patients, and was the endemic strain in wards 5 and 6, RVH. This S-type correlates with PCR ribotype 1 which is the

endemic strain in England and Wales (Brazier, 1998). S-type 5236/T was also the most frequently isolated type from the environment.

This study sought to determine if particular S-types were associated with asymptomatic colonisation and if others were associated with disease. Due to the high frequency of endemic S-type 5236/T and the small numbers of other S-types, it was not possible to compare symptoms between patients with different S-types. Patients colonised with more than one strain of *C. difficile* were also investigated to determine if they suffered from more episodes or a longer duration of disease. The statistical analysis found that colonisation with more than one S-type was significant in the development of symptoms, however not on severity of diarrhoea.

The location of the S-layer proteins on the bacterial cell surface make them ideal candidates for host-pathogen interactions and it is highly probable that they do play a role in virulence by influencing adherence, association with the mucosa or avoidance of the immune system. As well as the high degree of variation in the molecular mass of the S-layer proteins, this study showed the S-layer proteins to be immunogenic in rabbits. Western blotting showed that the heavier protein exhibited antigenic similarities between strains, whereas the lighter protein was more antigenically distinct between strains.

Further studies on *C. difficile* should aim to determine the risk factors which most influence colonisation and disease, the mechanisms by which antibiotics and other risk factors may exhibit their effects by possible up-regulation of toxins, and other virulence factors should be investigated. Potential virulence factors including the S-layer proteins, flagella and capsules should be examined to determine their role if any, in the pathogenesis of *C. difficile*.

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APPENDIX 1

Culture Media and Buffers

Media Formulations

All culture media were autoclaved at 121°C for 15 min before the addition of selective agents, blood and/or egg yolk emulsion.

Cycloserine-Cefoxitin, Egg-Yolk Agar (CCEY) for the isolation of *C. difficile* (adapted from Brazier, 1993)

Fastidious anaerobe agar (Lab M, Bury, England)	23 g
Cholic acid sodium salt (Sigma Chemicals, USA)	0.5 g
Cefoxitin (Sigma Chemicals)	8 μg/ml
Cycloserine (Sigma Chemicals)	250 μg/ml
Egg-yolk emulsion (Oxoid, Basingstoke, Hampshire)	20 ml
Lysed horse blood (Oxoid)	5 ml
Distilled water	500 ml

Cooked meat broth (CMB) Medium
(Collee and Marr, 1996)

Add 4 ml AIM to 0.5 cm^3 cooked meat particles (see below for preparation) and autoclave at 121°C/15 min.

Preparation of Cooked Meat particles

All fatty material was removed from 500 g fresh sheep hearts. The meat was then minced and added to 500 ml of distilled water containing 1.5 ml of 1 M NaOH solution and simmered for 20 min. The liquid was then drained off and dried at 60°C and particles were stored at –20°C until required.

Appendix 1

Anaerobic investigation medium (AIM)
(Brown et al, 1996)

Proteose peptone (Oxoid)	20 g
Yeast Extract (Oxoid)	5 g
Trypticase (BBL, Becton Dickinson, USA)	5 g
NaCl	5 g
Cysteine HCl	750 mg
Na_2CO_3	400 mg
Haemin (250μg/l) & Menadione (100μg/l) solution	20 ml
Distilled Water	1000 ml

Fastidious anaerobe broth supplemented with a modified *C. difficile* supplement

Fastidious anaerobe broth (Lab M)	23 g
Cholic acid sodium salt (Sigma)	0.5 g
Modified *C. difficile* supplement (Oxoid, SR173E)	1 vial*
Distilled water	500 ml

*1 vial of supplement contains 250 mg cysteine hydrochloride, 6 mg Norfloxacin and 16 mg Moxalactam.

Brain heart infusion/proteose peptone medium (BHI/PP)-*C. difficile* toxin production medium (Brettle et al, 1982)

Brain heart infusion broth (Oxoid)	37g
Proteose Peptone (Oxoid)	10g
Distilled water	1000 ml

Proteose peptone yeast (PPY) medium
(Deacon et al, 1978- adapted by Poxton et al, 1984)

Proteose peptone (Oxoid)	20g
Yeast extract (Difco Laboratories, USA)	10g
NaCl	5g
Cysteine HCl	750 mg
Na_2CO_3	400 mg
Haemin (250µg/l) & Menadione (100µg/l) solution	20 ml

BUFFERS.

Phosphate buffered saline (PBS).

Dulbecco A tablets (Oxoid BR14), one tablet added to 100 ml of distilled water.

POLYACRYLAMIDE GEL ELECTROPHORESIS BUFFERS
(Laemmli, 1970)

Double strength separating gel buffer [0.75 M Tris/HCl, pH 8.8, 0.2% sodium lauryl (dodecyl) sulphate (SDS)].

Tris (hydroxymethyl) methylamine	91 g
SDS	2 g
Pyrogen free water	1000 ml

Double strength stacking Buffer (0.25M Tris/HCl, pH 6.8, 0.2% SDS).

Tris (hydroxymethyl) methylamine	15 g
SDS	1 g
Pyrogen free water	500 ml

SDS-PAGE Electrode Buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3)

Tris (hydroxymethyl) methylamine	6 g
Glycine (chromatographically homogeneous)	29 g
SDS	2g
Pyrogen-free water	2000 ml

Double Strength sample buffer (0.125M Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.002% bromophenol blue)

Tris (hydroxymethyl) methylamine	1.5 g
SDS	4 g
Glycerol (20% v/v)	25 g
2-mercaptoethanol	2 ml
Bromophenol blue (0.05% aqueous solution)	4 ml
Pyrogen free water	100 ml

IMMUNOBLOTTING BUFFERS

Western blotting electrode buffer (0.025M Tris, 0.192M glycine, 20% methanol, pH 8.3)

Tris (hydroxymethyl) methylamine	6 g
Glycine	29 g
Methanol	400 ml
Distilled water	1600 ml

Tris buffered saline (TBS) [0.02M Tris/HCl, 0.5M NaCl, pH 7.5]

Tris (hydroxymethyl) methylamine	4.8 g
Sodium chloride	58.5 g
Distilled water	2000 ml

Tween Tris buffered saline (0.02M Tris/HCl, 0.5M NaCl, pH 7.5)

Tris (hydroxymethyl) methylamine	4.5 g
Sodium chloride	58.5 g
Tween 200	5 ml
Distilled water	2000 ml

Blocking Solution

(3% gelatin in TBS)

Gelatin (Bio-Rad USA, EIA grade)	22.5 g
TBS	750 ml

Antibody buffer

(1% gelatin in TBS)

Gelatin (Bio-Rad)	15 g
TBS	1500 ml

Horseradish peroxidase (HRP) colour development solution

Solution A

4-chloro-1-naphthol	60 mg
Methanol	20 ml

Solution B

Hydrogen peroxide	60µl
TBS	100 ml

Solutions A and B were freshly prepared and mixed just prior to use.

Coomassie Blue Stain
(Hancock and Poxton, 1988)

SDS-PAGE protein gels were stained overnight in Coomassie blue solution 1, de-staining was carried out using each of solutions 2-5 for one 1 h.

Staining solution 1

Coomassie brilliant blue R-250	1000 mg
Propan-2-ol	500 ml
Acetic Acid	200 ml
Distilled water	1300 ml

Staining solution 2

Coomassie brilliant blue R-250	100 mg
Propan-2-ol	200 ml
Acetic Acid	200 ml
Distilled water	1600 ml

Staining solution 3

Coomassie brilliant blue R-250	48 mg
Acetic Acid	200 ml
Distilled water	1800 ml

Staining solution 4

Methanol	800 ml
Acetic Acid	200 ml
Distilled water	1000 ml

Staining solution 5

Acetic Acid	200 ml
Distilled water	1800 ml

APPENDIX 2

A list of the strains supplied by Dr, J Brazier, ARU (mprl 4196-4219) and the clinical isolates collected from patients in wards 5 and 6, The Royal Victoria hospital, Edinburgh.

(The patient number, the S-type and the ability of isolates to produce toxin are listed)

Mprl isolates

Isolate number	Serotype	Ribotype
mprl 4196	A	
mprl 4197	B	
mprl 4198	C	
mprl 4199	D	
mprl 4200	F	
mprl 4201	G	
mprl 4202	H	
mprl 4203	I	
mprl 4204	K	
mprl 4205	X	
mprl 4206	A2	
mprl 4207	AA3	
mprl 4208	A4	
mprl 4209	A5	
mprl 4210	A6	
mprl 4211	A7	
mprl 4212	A8	
mprl 4213	A9	
mprl 4214	A10	
mprl 4215		10
mprl 4216		23
mprl 4217		26
mprl 4218		106
mprl 4219		56

Appendix 2

RVH clinical isolates

Isolate number	Patient number	S-type	In vitro toxin A and/or B production
203a	52	5236	Yes
205a	59	5242	Yes
210a	52	5236	Yes
216a	89	5242	Yes
218a	49	UT	Yes
222a	18	5236	No
223a	81	5236	Yes
224a	76	5236	Yes
226a	86	5236	Yes
239a	59	5242	Yes
244a	49	UT	Yes
246a	101	5236	Yes
247a	18	5236	Yes
253a	76	5236	Yes
257a	61	5236	Yes
261a	81	5236	No
265a	79	5236	Yes
269a	81	5236	No
271a	79	5236	Yes
274a	47	5236	Yes
277a	49	UT	Yes
280a	76	5236	Yes
281a	101	5236	Yes
289a	59	5242	Yes
310a	84	5236	Yes
316a	103	5236	Yes
322a	84	5236	Yes
332c	137	5236	No
334b	64	5236	Yes
335a	18	5236	Yes
336b	84	5236	Yes
338a	93	5236	Yes
346a	61	5236	Yes
348a	76	5236	Yes
357a	138	5236	Yes
359a	151	5043	Yes
360	93	5236	Yes
362a	76	5236	Yes
363a	81	5236	No
364a	84	5236	Yes

Appendix 2

Isolate number	Patient number	S-type	In vitro toxin A and/or B production
366a	144	5236	Yes
370a	68	5236	Yes
371a	82	5236	Yes
375a	143	5236	Yes
381a	93	5236	Yes
386a	76	5236	Yes
391a	84	5236	Yes
392a	82	5236	Yes
394b	103	5236	Yes
399a	172	5236	Yes
401a	76	5236	Yes
402	170	5236	Yes
408b	57	5236	Yes
414a	170	5236	Yes
419a	144	5236	Yes
426	201	5242	Yes
428	147	5236	Yes
431b	137	5236	Yes
432b	57	5242	Yes
433a	49	5236	Yes
439a	76	5236	Yes
440a	49	5236	Yes
444a	119	5236	Yes
445a	84	5236	Yes
447a	163	5236	Yes
461a	144	5236	Yes
467a	103	5236	Yes
473a	201	5242	Yes
480a	144	5236	Yes
483a	182	5236	Yes
484	205	5236	Yes
485	76	5236	Yes
487	93	5236	Yes
495a	76	5236	Yes
501a	93	5236	Yes
511a	201	5242	Yes
518a	170	5236	Yes
520a	233	5236	Yes
524a	151	5236	Yes
528a	250	5236	Yes
530a	93	5236	Yes
532a	449	5242	Yes
541a	240	5941	Yes
545a	93	5236	Yes
551a	233	5236	Yes
554	251	5236	Yes

Appendix 2

Isolate number	Patient number	S-type	In vitro toxin A and/or B production
556a	144	5236	Yes
561	251	5236	Yes
563a	367	5242	Yes
564a	170	5236	Yes
565	18	5236	Yes
566a	251	5236	Yes
568a	233	5236	Yes
580b	170	5236	Yes
584c	222	5236	Yes
588a	254	5236	Yes
589a	93	5236	Yes
602a	79	5236	Yes
605a	251	5236	Yes
621	18	5236	Yes
622	251	5236	Yes
623a	170	5236	Yes
625a	325	5144	No
628a	337	5144	No
633a	251	5236	Yes
638a	316	5236	Yes
643a	303	5236	Yes
659a	367	5242	Yes
661a	316	5236	Yes
662c	206	5236	Yes
666a	206	5236	Yes
667a	366	5236	Yes
668a	361	5236	Yes
669a	367	5236	Yes
672a	337	5236	Yes
679a	366	5242	Yes
681a	361	5236	Yes
697	367	5236	Yes
702a	366	5236	Yes
703	345	5236	Yes
704	251	5236	Yes
705a	367	5242	Yes
708b	366	5236	Yes
711a	402	5236	Yes
712b	411	5236	Yes
729	414	5236	Yes
748a	402	5236	No
749a	411	5236	Yes
750a	409	5236	Yes
751a	368	5236	Yes
753a	347	5236	Yes
754a	361	5236	Yes

Appendix 2

Isolate number	Patient number	S-type	In vitro toxin A and/or B production
758a	426	5236	Yes
767	346	5236	Yes
778a	449	5242	Yes
779a	427	5236	Yes
780a	398	5236	Yes
781a	427	5236	Yes
784	306	5144	No
788a	368	5236	Yes
790a	354	5236	Yes
793a	368	5236	Yes
797a	467	5236	Yes
803a	458	5438	Yes
804c	378	5438	Yes
811a	335	5236	Yes
813a	435	5236	Yes
814a	467	5236	Yes
816a	468	5236	No
827a	411	5236	Yes
845a	560	5236	Yes
852a	472	5236	Yes
859a	472	5236	Yes
896a	537	5236	Yes
898a	560	5236	Yes
900a	498	5236	Yes
918a	579	5242	Yes
922a	537	5144	No
926a	567	5236	Yes
934a	562	5236	Yes
935a	515	5236	Yes
936	577	5140	Yes
937a	564	5144	No
945a	567	5236	Yes
958	603	5242	Yes
959a	560	5236	Yes
960a	567	5242	Yes
961a	604	5242	Yes
964a	609	5242	Yes
965a	553	5236	Yes
967a	597	5140	Yes
969b	597	5140	Yes
972a	609	5242	Yes
973a	591	5236	Yes
981a	553	5236	Yes
982	597	5140	Yes
983a	534	5236	Yes
986a	510	5236	Yes

Appendix 2

Isolate number	Patient number	S-type	In vitro toxin A and/or B production
988a	610	5242	Yes
990a	627	5236	Yes
992d	469	5438	Yes
996a	596	5242	Yes

APPENDIX 3
ENVIRONMENTAL SAMPLES: The date, sampling method, ward and area sampled, and the result
(N= negative for *C. difficile*; P= positive for *C. difficile*)

Date of sampling	Sampling method	Ward	Area sampled			Result
06-Aug-99	contact plate	5	bay 4	toilet area	floor	N
06-Aug-99	contact plate	5	bay 3	toilet area	commode	N
06-Aug-99	contact plate	5	bay 2	toilet area	commode	N
06-Aug-99	contact plate	5	bay 4	main bay	floor	N
06-Aug-99	contact plate	5	bay 4	toilet area	floor	N
06-Aug-99	contact plate	5	shower-room		shower seat	N
06-Aug-99	contact plate	5	bathroom		bath	N
06-Aug-99	contact plate	5	bathroom		ambulift	N
06-Aug-99	contact plate	5	bay 1	main bay	floor	N
06-Aug-99	contact plate	5	bay 1	main bay	floor	N
06-Aug-99	contact plate	5	bay 2	main bay	floor	N
06-Aug-99	contact plate	5	bay 2	toilet area	floor	N
06-Aug-99	contact plate	5	bay 3	main bay	floor	N
06-Aug-99	contact plate	5	shower-room		shower floor	N
06-Aug-99	swab	5	bathroom		sitting scales	N
06-Aug-99	swab	5	bay 3	toilet area	commode handle	N
06-Aug-99	swab	5	bay 4	main bay	commode	N
06-Aug-99	swab	5	sluice-room		bed pan disposal unit	N
06-Aug-99	swab	5	bay 1	toilet area	toilet handle	N
06-Aug-99	swab	5	bay 1	toilet area	toilet door handle	N
06-Aug-99	swab	5	bay 2	toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
06-Aug-99	swab	5	bay 2	toilet area	toilet door handle	N
06-Aug-99	swab	5	bay 3	toilet area	toilet handle	N
06-Aug-99	swab	5	bay 3	toilet area	toilet door handle	N
06-Aug-99	swab	5	bay 4	toilet area	toilet handle	N
06-Aug-99	swab	5	bay 4	toilet area	toilet door handle	N
06-Aug-99	swab	5	bay 2	main bay	wash basin tap handle	N
06-Aug-99	swab	5	bay 3	main bay	wash basin tap handle	N
06-Aug-99	contact plate	6	SR 6	main room	floor	N
06-Aug-99	contact plate	6	bay 4	toilet area	toilet	N
06-Aug-99	contact plate	6	bay 4	main bay	floor	N
06-Aug-99	contact plate	6	bay 3	toilet area	toilet	N
06-Aug-99	contact plate	6	bay 3	main bay	floor	N
06-Aug-99	contact plate	6	bay 1	toilet area	toilet	N
06-Aug-99	contact plate	6	bay 2	main bay	floor	N
06-Aug-99	contact plate	6	shower-room		shower floor	N
06-Aug-99	contact plate	6	bathroom		ambulift	N
06-Aug-99	contact plate	6	bathroom		floor	N
06-Aug-99	contact plate	6	sluice-room		commode	N
06-Aug-99	contact plate	6	sluice-room		commode	N
06-Aug-99	contact plate	6	bay 2	toilet area	toilet	N
06-Aug-99	contact plate	6	bay 1	main bay	floor	N
06-Aug-99	swab	6	bay 4	main bay	wash basin tap handle	N
06-Aug-99	swab	6	sluice-room		bed pan disposal unit	N

Date of sampling	Sampling method	Ward	Area sampled			Result
06-Aug-99	swab	6	sluice-room		commode	N
06-Aug-99	swab	6	bay 1	toilet area	toilet handle	N
06-Aug-99	swab	6	bay 1	toilet area	toilet door handle	N
06-Aug-99	swab	6	bay 2	toilet area	toilet handle	N
06-Aug-99	swab	6	bay 2	toilet area	toilet door handle	N
06-Aug-99	swab	6	bay 3	toilet area	toilet handle	N
06-Aug-99	swab	6	bay 3	toilet area	toilet door handle	N
06-Aug-99	swab	6	bay 4	toilet area	toilet handle	N
06-Aug-99	swab	6	bay 4	toilet area	toilet door handle	N
06-Aug-99	swab	6	bay 2	main bay	wash basin tap handle	N
06-Aug-99	swab	6	bathroom		toilet handle	N
25-Aug-99	contact plate	5	bay 2	main bay	floor	N
25-Aug-99	contact plate	5	bay 2	main bay	commode	N
25-Aug-99	contact plate	5	bay 3	main bay	floor	N
25-Aug-99	contact plate	5	bay 3	toilet area	floor	N
25-Aug-99	contact plate	5	bay 4	main bay	floor	N
25-Aug-99	contact plate	5	bay 1	main bay	floor	N
25-Aug-99	contact plate	5	bathroom		bath	N
25-Aug-99	contact plate	5	bay 1	toilet area	toilet seat	N
25-Aug-99	contact plate	5	shower-room		floor	N
25-Aug-99	contact plate	5	bay 1	main bay	floor	N
25-Aug-99	swab	5	bay 4	main bay	wash basin tap handle	N
25-Aug-99	swab	5		main bay	hoist sling	N
25-Aug-99	swab	5	bay 1	toilet area	toilet seat	N

Date of sampling	Sampling method	Ward	Area sampled			Result
25-Aug-99	swab	5	sluice-room		bed pan disposal unit	N
25-Aug-99	swab	5	bay 1	toilet area	toilet handle	N
25-Aug-99	swab	5	bay 4	toilet area	wash basin tap handle	N
25-Aug-99	swab	5	bay 4	toilet area	toilet handle	N
25-Aug-99	swab	5	bay 1	toilet area	wash basin tap handle	N
25-Aug-99	swab	5	bay 1	toilet area	toilet door handle	N
25-Aug-99	swab	5	bay 2	toilet area	toilet door handle	N
25-Aug-99	swab	5	bay 2	main bay	commode	N
25-Aug-99	swab	5	bay 2	main bay	commode	N
25-Aug-99	swab	5	bay 2	main bay	wash basin tap handle	N
25-Aug-99	swab	5	bay 3	toilet area	toilet door handle	N
25-Aug-99	swab	5	bay 3	main bay	commode	N
25-Aug-99	contact plate	6	bathroom		ambulift	N
25-Aug-99	contact plate	6	shower-room		shower seat	N
25-Aug-99	contact plate	6	sluice-room		commode	N
25-Aug-99	contact plate	6	bay 1	main bay	floor	N
25-Aug-99	contact plate	6	bay 1	toilet area	floor	N
25-Aug-99	contact plate	6	bay 2	main bay	floor	N
25-Aug-99	contact plate	6	bay 3	main bay	floor	N
25-Aug-99	contact plate	6	bay 3	toilet area	toilet seat	N
25-Aug-99	contact plate	6	shower-room		floor	N
25-Aug-99	swab	6	bay 2	toilet area	toilet door handle	N
25-Aug-99	swab	6	bay 2	main bay	wash basin tap handle	N
25-Aug-99	swab	6	bay 3	toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
25-Aug-99	swab	6	bay 3	toilet area	toilet seat	N
25-Aug-99	swab	6	bay 4	toilet area	toilet seat	N
25-Aug-99	swab	6	bay 4	toilet area	toilet door handle	N
25-Aug-99	swab	6	SR 6	toilet area	toilet handle	N
25-Aug-99	swab	6	SR 6	toilet area	toilet handle	N
25-Aug-99	swab	6	bay 4	main bay	wash basin tap handle	N
25-Aug-99	swab	6	sluice-room		bed pan disposal unit	N
25-Aug-99	swab	6	sluice-room		commode	N
25-Aug-99	swab	6	bay 1	toilet area	toilet door handle	N
25-Aug-99	swab	6		main bay	hoist sling	N
25-Aug-99	swab	6	bay 1	main bay	wash basin tap handle	N
01-Sep-99	contact plate	5	bay 1	toilet area	floor	N
01-Sep-99	contact plate	5	bay 4	main bay	floor	N
01-Sep-99	contact plate	5	bay 4	main bay	floor	N
01-Sep-99	contact plate	5	SR 8	main room	floor	N
01-Sep-99	contact plate	5	bay 3	toilet area	floor	N
01-Sep-99	contact plate	5	bathroom		ambulift	N
01-Sep-99	contact plate	5	shower-room		floor	N
01-Sep-99	contact plate	5	bay 3	main bay	floor	N
01-Sep-99	contact plate	5	bay 2	main bay	commode	N
01-Sep-99	contact plate	5	bay 2	main bay	floor	N
01-Sep-99	contact plate	5	bay 1	main bay	floor	N
01-Sep-99	contact plate	5	Bathroom		bath	N
01-Sep-99	contact plate	5	shower-room		shower seat	N

Date of sampling	Sampling method	Ward	Area sampled			Result
01-Sep-99	contact plate	5	bay 3	toilet area	toilet seat	N
01-Sep-99	swab	5	bay 1	toilet area	toilet handle	N
01-Sep-99	swab	5	bay 2	toilet area	toilet handle	N
01-Sep-99	swab	5	sluice-room		bed pan disposal unit	N
01-Sep-99	swab	5	bay 1	toilet area	toilet handle	N
01-Sep-99	swab	5	bay 1	toilet area	wash basin tap handle	N
01-Sep-99	swab	5	bay 3	toilet area	toilet door handle	N
01-Sep-99	swab	5	bay 4	toilet area	toilet handle	N
01-Sep-99	swab	5	bay 3	toilet area	wash basin tap handle	N
01-Sep-99	swab	5	bay 3	toilet area	toilet seat	N
01-Sep-99	swab	5	bay 3	toilet area	toilet handle	N
01-Sep-99	swab	5	bay 2	toilet area	toilet door handle	N
01-Sep-99	swab	5	bay 2	toilet area	toilet seat	N
01-Sep-99	swab	5	bay 1	toilet area	commode	N
01-Sep-99	swab	5	bay 4	toilet area	toilet handle	N
01-Sep-99	contact plate	6	bay 1	toilet area	toilet seat	N
01-Sep-99	contact plate	6	SR 6	toilet area	floor	N
01-Sep-99	contact plate	6	SR 6	main room	floor	N
01-Sep-99	contact plate	6	bay 4	main bay	floor	N
01-Sep-99	contact plate	6	bay 3	main bay	floor	N
01-Sep-99	contact plate	6	bay 2	toilet area	floor	N
01-Sep-99	contact plate	6	bay 1	main bay	floor	N
01-Sep-99	contact plate	6	bay 1	main bay	floor	N
01-Sep-99	contact plate	6	shower-room		shower seat	N

Date of sampling	Sampling method	Ward	Area sampled			Result
01-Sep-99	contact plate	6	shower-room		floor	N
01-Sep-99	contact plate	6	bathroom		bath	N
01-Sep-99	contact plate	6	bathroom		ambulift	N
01-Sep-99	contact plate	6	sluice-room		commode	N
01-Sep-99	contact plate	6	bay 2	main bay	floor	N
01-Sep-99	swab	6		main bay	stand-aid sling	N
01-Sep-99	swab	6	bay 3	toilet area	toilet handle	N
01-Sep-99	swab	6	bay 3	toilet area	toilet seat	N
01-Sep-99	swab	6	bay 4	toilet area	toilet door handle	N
01-Sep-99	swab	6		main bay	maxi-hoist sling	N
01-Sep-99	swab	6	bay 4	toilet area	toilet handle	N
01-Sep-99	swab	6	sluice-room		commode	N
01-Sep-99	swab	6	bay 2	toilet area	toilet handle	N
01-Sep-99	swab	6	bay 1	toilet area	toilet seat	N
01-Sep-99	swab	6	bay 1	toilet area	toilet door handle	N
01-Sep-99	swab	6	bay 1	toilet area	wash basin tap handle	N
01-Sep-99	swab	6	bay 1	toilet area	toilet handle	N
01-Sep-99	swab	6	sluice-room		commode	N
01-Sep-99	swab	6	sluice-room		bed pan disposal unit	N
01-Sep-99	swab	6	bay 2	toilet area	toilet handle	N
22-Sep-99	contact plate	5	shower-room		shower floor	N
22-Sep-99	contact plate	5	shower-room		shower seat	N
22-Sep-99	contact plate	5	bay 1	main bay	floor	N
22-Sep-99	contact plate	5	bay 1	toilet area	floor	N

Date of sampling	Sampling method	Ward	Area sampled			Result
22-Sep-99	contact plate	5	bay 3	main bay	floor	N
22-Sep-99	contact plate	5	bay 3	toilet area	toilet seat	N
22-Sep-99	contact plate	5	SR 8	main room	floor	N
22-Sep-99	contact plate	5	SR 8	toilet area	toilet floor	N
22-Sep-99	contact plate	5	SR 8	toilet area	commode	N
22-Sep-99	contact plate	5	bathroom		bath	N
22-Sep-99	swab	5	bay 3	toilet area	commode	N
22-Sep-99	swab	5	SR 8	main room	commode	N
22-Sep-99	swab	5	SR 8	toilet area	toilet handle	N
22-Sep-99	swab	5	SR 8	toilet area	toilet door handle	N
22-Sep-99	swab	5	SR 8	toilet area	toilet handle	N
22-Sep-99	swab	5			transport hoist sling	N
22-Sep-99	swab	5	bay 3	toilet area	toilet door handle	N
22-Sep-99	swab	5	bay 3	toilet area	wash basin tap handle	N
22-Sep-99	swab	5	bay 3	toilet area	toilet handle	N
22-Sep-99	swab	5	bay 1	toilet area	commode	N
22-Sep-99	swab	5	bay 1	toilet area	wash basin tap handle	N
22-Sep-99	swab	5	bay 1	toilet area	toilet handle	N
22-Sep-99	swab	5	bay 1	toilet area	toilet handle	N
22-Sep-99	swab	5	sluice-room		bed pan disposal unit	N
22-Sep-99	contact plate	6	bay 3	main bay	floor	N
22-Sep-99	contact plate	6	sluice-room		commode	N
22-Sep-99	contact plate	6	shower-room		shower floor	N
22-Sep-99	contact plate	6	bay 3	toilet area	floor	N

Date of sampling	Sampling method	Ward	Area sampled			Result
22-Sep-99	contact plate	6	bay 3	toilet area	toilet seat	N
22-Sep-99	contact plate	6	bay 3	main bay	floor	N
22-Sep-99	contact plate	6	SR 9	main room	floor	P
22-Sep-99	contact plate	6	SR 9	toilet area	toilet seat	P
22-Sep-99	contact plate	6	SR 6	main room	floor	P
22-Sep-99	contact plate	6	SR 6	toilet area	floor	P
22-Sep-99	contact plate	6	SR 7	toilet area	floor	N
22-Sep-99	contact plate	6	SR 7	main room	floor	N
22-Sep-99	contact plate	6	bay 2	main bay	floor	N
22-Sep-99	contact plate	6	bathroom		bath	N
22-Sep-99	swab	6	SR 7	toilet area	toilet handle	N
22-Sep-99	swab	6	SR 6	toilet area	tap handle	N
22-Sep-99	swab	6	SR 6	toilet area	toilet seat	N
22-Sep-99	swab	6	SR 6	toilet area	toilet handle	N
22-Sep-99	swab	6	SR 9	toilet area	tap handle	N
22-Sep-99	swab	6	SR 9	toilet area	toilet seat	N
22-Sep-99	swab	6	SR 9	toilet area	toilet handle	N
22-Sep-99	swab	6	bay 3	main bay	wash basin tap handle	N
22-Sep-99	swab	6	bay 3	toilet area	toilet door handle	N
22-Sep-99	swab	6		main bay	transport hoist sling	N
22-Sep-99	swab	6	bay 3	toilet area	toilet handle	N
22-Sep-99	swab	6		main bay	transport hoist sling	N
22-Sep-99	swab	6	bay 3	toilet area	toilet seat	N
22-Sep-99	swab	6	SR 7	main room	commode	N

Date of sampling	Sampling method	Ward	Area sampled			Result
30-Sep-99	contact plate	5	bay 2	toilet area	floor	N
30-Sep-99	contact plate	5	SR 8	toilet area	toilet floor	N
30-Sep-99	contact plate	5	SR 9	main room	floor	N
30-Sep-99	contact plate	5	SR 8	main room	commode	N
30-Sep-99	contact plate	5	SR 8	main room	floor	N
30-Sep-99	contact plate	5	SR 9	toilet area	floor	N
30-Sep-99	contact plate	5	bay 3	main bay	floor	N
30-Sep-99	contact plate	5	bay 2	main bay	floor	N
30-Sep-99	contact plate	5	shower-room		shower seat	N
30-Sep-99	contact plate	5	shower-room		shower floor	N
30-Sep-99	contact plate	5	bathroom		bath	N
30-Sep-99	contact plate	5	bay 1	toilet area	commode	N
30-Sep-99	contact plate	5	bay 1	toilet area	floor	N
30-Sep-99	contact plate	5	bay 1	main bay	floor	N
30-Sep-99	swab	5	SR 9	toilet area	commode	N
30-Sep-99	swab	5	SR 8	main room	commode	N
30-Sep-99	swab	5	bay 3	toilet area	commode	P
30-Sep-99	swab	5	bay 3	toilet area	toilet door handle	N
30-Sep-99	swab	5	bay 3	toilet area	toilet handle	N
30-Sep-99	swab	5	bay 3	toilet area	toilet seat	N
30-Sep-99	swab	5	SR 8	toilet area	toilet door handle	N
30-Sep-99	swab	5	SR 8	toilet area	toilet handle	N
30-Sep-99	swab	5	bay 3	toilet area	toilet door handle	N
30-Sep-99	swab	5	bay 3	toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
30-Sep-99	swab	5	bay 2	toilet area	toilet door handle	N
30-Sep-99	swab	5	bay 2	toilet area	toilet handle	N
30-Sep-99	swab	5	SR 9	toilet area	toilet door handle	N
30-Sep-99	swab	5	SR 9	toilet area	toilet handle	N
30-Sep-99	swab	5	bay 3	toilet area	wash basin tap handle	N
30-Sep-99	swab	5	bay 3	toilet area	toilet handle	N
30-Sep-99	swab	5	sluice-room		bed pan disposal unit	N
30-Sep-99	swab	5	sluice-room		bed pan disposal unit	N
30-Sep-99	swab	5	bay 3	toilet area	toilet door handle	N
30-Sep-99	contact plate	6	SR 9	toilet area	floor	P
30-Sep-99	contact plate	6	SR 9	main room	floor	P
30-Sep-99	contact plate	6	SR 7	main room	floor	P
30-Sep-99	contact plate	6	SR 6	toilet area	toilet seat	N
30-Sep-99	contact plate	6	SR 6	toilet area	floor	P
30-Sep-99	contact plate	6	SR 6	main room	floor	P
30-Sep-99	contact plate	6	bay 3	toilet area	toilet seat	N
30-Sep-99	contact plate	6	bay 3	toilet area	door	N
30-Sep-99	contact plate	6	bay 3	main bay	floor	P
30-Sep-99	contact plate	6	shower-room		shower seat	N
30-Sep-99	contact plate	6	shower-room		shower floor	N
30-Sep-99	contact plate	6	SR 10	toilet area	Commode	N
30-Sep-99	contact plate	6	bathroom		bath	N
30-Sep-99	contact plate	6	SR 9	toilet area	commode	P
30-Sep-99	contact plate	6	SR 10	main room	floor	N

Date of sampling	Sampling method	Ward	Area sampled			Result
30-Sep-99	contact plate	6	bay 4	main bay	floor	N
30-Sep-99	swab	6	SR 9	main room	commode	N
30-Sep-99	swab	6	SR 9	toilet area	toilet seat	N
30-Sep-99	swab	6	SR 9	toilet area	toilet handle	P
30-Sep-99	swab	6	SR 6	toilet area	toilet door handle	N
30-Sep-99	swab	6	SR 6	main room	wash basin tap handle	N
30-Sep-99	swab	6	SR 6	toilet area	toilet handle	N
30-Sep-99	swab	6	bay 3	main bay	wash basin tap handle	N
30-Sep-99	swab	6	bay 4	toilet area	toilet handle	N
04-Oct-99	contact plate	5	bathroom		bath	N
04-Oct-99	contact plate	5	bay 4	main bay	floor	N
04-Oct-99	contact plate	5	bay 3	toilet area	floor	N
04-Oct-99	contact plate	5	SR 8	main room	commode	N
04-Oct-99	contact plate	5	SR 8	toilet area	toilet floor	N
04-Oct-99	contact plate	5	SR 8	main room	floor	N
04-Oct-99	contact plate	5	bay 2	toilet area	commode	N
04-Oct-99	contact plate	5	bay 2	toilet area	floor	N
04-Oct-99	contact plate	5	bay 2	main bay	floor	N
04-Oct-99	contact plate	5	bay 1	toilet area	commode	N
04-Oct-99	contact plate	5	bay 1	toilet area	floor	N
04-Oct-99	contact plate	5	shower-room		shower floor	N
04-Oct-99	contact plate	5	shower-room		shower seat	N
04-Oct-99	contact plate	5	bay 1	main bay	floor	N
04-Oct-99	swab	5	bay 1	toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
04-Oct-99	swab	5	sluice-room		bed pan disposal unit	N
04-Oct-99	swab	5	SR 8	main room	Wash basin tap handle	N
04-Oct-99	swab	5	bay 3	toilet area	toilet door handle	N
04-Oct-99	swab	5	SR 8	toilet area	toilet door handle	N
04-Oct-99	swab	5	SR 8	main room	Commode handle	N
04-Oct-99	swab	5	SR 8	toilet area	toilet handle	N
04-Oct-99	swab	5	bay 2	toilet area	commode handle	N
04-Oct-99	swab	5	bay 2	toilet area	toilet door handle	N
04-Oct-99	swab	5	bay 2	toilet area	toilet handles	N
04-Oct-99	swab	5	bay 1	toilet area	commode handle	N
04-Oct-99	swab	5	bay 3	toilet area	toilet handles	N
04-Oct-99	swab	5	bay 3	toilet area	toilet seat	N
04-Oct-99	swab	5	bay 1	toilet area	toilet door handle	N
04-Oct-99	contact plate	6	shower-room		floor	N
04-Oct-99	contact plate	6	bay 4	main bay	floor	N
04-Oct-99	contact plate	6	SR 10	main room	floor	P
04-Oct-99	contact plate	6	SR 9	main room	floor	P
04-Oct-99	contact plate	6	SR 7	toilet area	toilet floor	P
04-Oct-99	contact plate	6	SR 7	main room	floor	N
04-Oct-99	contact plate	6	SR 6	toilet area	toilet floor	P
04-Oct-99	contact plate	6	SR 6	main room	floor	N
04-Oct-99	contact plate	6	bay 3	toilet area	commode	P
04-Oct-99	contact plate	6	bay 3	toilet area	toilet seat	P
04-Oct-99	contact plate	6	bay 3	toilet area	floor	P

Date of sampling	Sampling method	Ward	Area sampled			Result
04-Oct-99	contact plate	6	shower-room		shower seat	N
04-Oct-99	contact plate	6	SR 10	toilet area	toilet floor	P
04-Oct-99	contact plate	6	bathroom		bath	N
04-Oct-99	contact plate	6	bay 3	main bay	floor	N
04-Oct-99	contact plate	6	SR 9	toilet area	toilet floor	P
04-Oct-99	swab	6	bay 3	toilet area	toilet seat	N
04-Oct-99	swab	6	bay 3	toilet area	toilet handle	N
04-Oct-99	swab	6	bay 3	toilet area	toilet door handle	N
04-Oct-99	swab	6	sluice-room		bed pan disposal unit	N
04-Oct-99	swab	6	bay 4	toilet area	toilet door handle	N
04-Oct-99	swab	6	bay 3	main bay	wash basin tap handle	N
04-Oct-99	swab	6	bay 4	toilet area	toilet handle	N
04-Oct-99	swab	6	SR 9	toilet area	toilet door handle	P
04-Oct-99	swab	6	SR 9	toilet area	toilet handle	N
04-Oct-99	swab	6	SR 6	toilet area	toilet door handle	P
04-Oct-99	swab	6	SR 6	toilet area	toilet seat	P
04-Oct-99	swab	6	SR 6	toilet area	toilet handle	N
04-Oct-99	swab	6	bay 3	toilet area	commode handle	N
04-Oct-99	swab	6	SR 9	toilet area	toilet seat	P
07-Oct-99	contact plate	5	SR 8	toilet area	floor	N
07-Oct-99	contact plate	5	bay 4	toilet area	floor	P
07-Oct-99	contact plate	5	SR 8	main room	Commode	N
07-Oct-99	contact plate	5	bay 2	toilet area	commode	P
07-Oct-99	contact plate	5	SR 8	main room	floor	N

Date of sampling	Sampling method	Ward	Area sampled			Result
07-Oct-99	contact plate	5	bay 2	toilet area	floor	N
07-Oct-99	contact plate	5	bay 1	toilet area	Commode	N
07-Oct-99	contact plate	5	bay 1	toilet area	floor	N
07-Oct-99	contact plate	5	bay 1	main bay	floor	N
07-Oct-99	contact plate	5	shower-room		shower seat	N
07-Oct-99	contact plate	5	shower-room		shower floor	N
07-Oct-99	contact plate	5	bathroom		bath	N
07-Oct-99	contact plate	5	bay 3	main bay	floor	N
07-Oct-99	contact plate	5	bay 2	main bay	floor	N
07-Oct-99	swab	5	bay 2	toilet area	toilet door handle	N
07-Oct-99	swab	5	bay 4	toilet area	toilet door handle	N
07-Oct-99	swab	5	bay 4	toilet area	toilet handles	N
07-Oct-99	swab	5	bay 3	toilet area	toilet seat	N
07-Oct-99	swab	5	bay 3	toilet area	toilet floor	N
07-Oct-99	swab	5	SR 8	main room	Commode handle	N
07-Oct-99	swab	5	SR 8	toilet area	toilet handles	N
07-Oct-99	swab	5	bay 2	toilet area	toilet seat	N
07-Oct-99	swab	5	SR 8	toilet area	toilet door handle	N
07-Oct-99	swab	5	bay 1	toilet area	toilet door handle	N
07-Oct-99	swab	5	bay 2	toilet area	toilet handles	N
07-Oct-99	swab	5	bay 1	toilet area	commode handle	N
07-Oct-99	swab	5	sluice-room		bed pan disposal unit	N
07-Oct-99	swab	5	bay 1	toilet area	toilet handles	N
07-Oct-99	contact plate	6	SR 9	main room	floor	P

Date of sampling	Sampling method	Ward	Area sampled			Result
07-Oct-99	contact plate	6	SR 6	toilet area	toilet floor	P
07-Oct-99	contact plate	6	SR 6	main room	floor	N
07-Oct-99	contact plate	6	bay 3	toilet area	toilet seat	P
07-Oct-99	contact plate	6	bay 3	toilet area	toilet floor	P
07-Oct-99	contact plate	6	bay 3	main bay	floor	P
07-Oct-99	contact plate	6	shower-room		shower seat	N
07-Oct-99	contact plate	6	shower-room		shower floor	N
07-Oct-99	contact plate	6	bathroom		bath	N
07-Oct-99	contact plate	6	SR 9	toilet area	toilet floor	N
07-Oct-99	contact plate	6	SR 9	toilet area	toilet seat	P
07-Oct-99	contact plate	6	SR 10	main room	floor	P
07-Oct-99	contact plate	6	SR 10	toilet area	floor	P
07-Oct-99	contact plate	6	bay 4	main bay	floor	P
07-Oct-99	swab	6		main bay	stand-aid sling	N
07-Oct-99	swab	6	bay 3	toilet area	toilet door handle	N
07-Oct-99	swab	6	bay 3	toilet area	commode handle	N
07-Oct-99	swab	6	bay 3	main bay	wash basin tap handle	N
07-Oct-99	swab	6	SR 6	toilet area	toilet handle	N
07-Oct-99	swab	6	SR 6	toilet area	toilet seat	N
07-Oct-99	swab	6	SR 9	toilet area	toilet handle	N
07-Oct-99	swab	6	bay 4	toilet area	toilet handles	N
07-Oct-99	swab	6	SR 6	toilet area	toilet door handle	N
07-Oct-99	swab	6	bay 3	toilet area	toilet seat	N
07-Oct-99	swab	6	SR 9	toilet area	toilet door handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
07-Oct-99	swab	6	bay 3	toilet area	toilet handle	N
07-Oct-99	swab	6	SR 9	toilet area	toilet seat	N
07-Oct-99	swab	6	sluice-room		bed pan disposal unit	N
12-Oct-99	contact plate	5	bay 1	toilet area	commode	N
12-Oct-99	contact plate	5	shower-room		shower floor	N
12-Oct-99	contact plate	5	shower-room		shower seat	N
12-Oct-99	contact plate	5	bay 1	main bay	floor	N
12-Oct-99	contact plate	5	bay 1	toilet area	floor	P
12-Oct-99	contact plate	5	bathroom		bath	N
12-Oct-99	contact plate	5	SR 8	main room	floor	N
12-Oct-99	contact plate	5	bay 3	main bay	floor	N
12-Oct-99	contact plate	5	bay 4	toilet area	toilet floor	N
12-Oct-99	contact plate	5	bay 4	main bay	floor	N
12-Oct-99	contact plate	5	SR 8	toilet area	toilet floor	N
12-Oct-99	contact plate	5	bay 2	toilet area	toilet floor	N
12-Oct-99	contact plate	5	sluice-room		bed pan disposal unit	N
12-Oct-99	contact plate	5	SR 8	main room	Commode	N
12-Oct-99	swab	5	SR 8	main room	Commode	N
12-Oct-99	swab	5	bay 1	toilet area	commode	N
12-Oct-99	swab	5	bay 1	toilet area	toilet door handle	N
12-Oct-99	swab	5	sluice-room		bed pan disposal unit	N
12-Oct-99	swab	5	bay 2	toilet area	commode	N
12-Oct-99	swab	5	bay 2	toilet area	toilet handle	N
12-Oct-99	swab	5	bay 1	toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
12-Oct-99	swab	5	bay 2	toilet area	toilet door handle	N
12-Oct-99	swab	5	SR 8	toilet area	toilet handle	N
12-Oct-99	swab	5	bay 3	toilet area	toilet handles	N
12-Oct-99	swab	5	bay 4	toilet area	toilet door handle	N
12-Oct-99	swab	5	bay 4	toilet area	toilet handles	N
12-Oct-99	swab	5	bay 3	toilet area	toilet seat	N
12-Oct-99	swab	5	SR 8	toilet area	toilet door handle	N
12-Oct-99	contact plate	6	bay 4	toilet area	floor	N
12-Oct-99	contact plate	6	bay 2	toilet area	toilet floor	N
12-Oct-99	contact plate	6	bay 1	toilet area	toilet floor	N
12-Oct-99	contact plate	6	SR 9	toilet area	toilet floor	N
12-Oct-99	contact plate	6	SR 9	main room	floor	P
12-Oct-99	contact plate	6	SR 6	toilet area	toilet floor	P
12-Oct-99	contact plate	6	SR 6	main room	floor	N
12-Oct-99	contact plate	6	bay 3	toilet area	toilet seat	P
12-Oct-99	contact plate	6	bay 3	toilet area	toilet floor	P
12-Oct-99	contact plate	6	SR 10	main room	floor	P
12-Oct-99	contact plate	6	bay 3	main bay	floor	P
12-Oct-99	contact plate	6	shower-room		shower seat	N
12-Oct-99	contact plate	6	shower-room		shower floor	N
12-Oct-99	contact plate	6	bathroom		bath	N
12-Oct-99	swab	6	SR 6	toilet area	toilet handle	P
12-Oct-99	swab	6	SR 6	toilet area	toilet seat	N
12-Oct-99	swab	6	bay 1	toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
12-Oct-99	swab	6	bay 1	toilet area	toilet door handle	N
12-Oct-99	swab	6	bay 4	toilet area	toilet handle	P
12-Oct-99	swab	6	bay 4	toilet area	toilet door handle	N
12-Oct-99	swab	6	SR 9	toilet area	toilet door handle	N
12-Oct-99	swab	6	SR 9	main room	commode	P
12-Oct-99	swab	6	bay 3	toilet area	commode	N
12-Oct-99	swab	6	SR 6	toilet area	toilet door handle	N
12-Oct-99	swab	6	bay 3	main bay	wash basin tap handle	N
12-Oct-99	swab	6	bay 3	toilet area	toilet door handle	N
12-Oct-99	swab	6	bay 3	toilet area	toilet seat	N
12-Oct-99	swab	6	bay 3	toilet area	toilet handle	P
12-Oct-99	swab	6	sluice-room		bed pan disposal unit	N
12-Oct-99	swab	6	SR 9	toilet area	toilet handle	P
20-Oct-99	contact plate	5	bay 1	toilet area	floor	P
20-Oct-99	contact plate	5	bay 4	toilet area	floor	N
20-Oct-99	contact plate	5	shower-room		shower floor	N
20-Oct-99	contact plate	5	bay 1	main bay	floor	N
20-Oct-99	contact plate	5	bathroom		bath	N
20-Oct-99	contact plate	5	bay 1	toilet area	commode	N
20-Oct-99	contact plate	5	SR 8	main room	floor	N
20-Oct-99	contact plate	5	SR 8	toilet area	floor	N
20-Oct-99	contact plate	5	SR 8	main room	commode	N
20-Oct-99	contact plate	5	bay 2	main bay	floor	N
20-Oct-99	contact plate	5	bay 2	toilet area	toilet seat	N

Date of sampling	Sampling method	Ward	Area sampled			Result
20-Oct-99	contact plate	5	bay 3	main bay	floor	N
20-Oct-99	contact plate	5	bay 3	toilet area	toilet seat	N
20-Oct-99	contact plate	5	shower-room		shower seat	P
20-Oct-99	swab	5	bay 1	toilet area	toilet handle	N
20-Oct-99	swab	5	bay 1	toilet area	commode	N
20-Oct-99	swab	5	bay 1	toilet area	toilet door handle	N
20-Oct-99	swab	5	sluice-room		bed pan disposal unit	N
20-Oct-99	swab	5	SR 8	toilet area	toilet handle	N
20-Oct-99	swab	5	bay 4	toilet area	toilet door handle	N
20-Oct-99	swab	5	bay 3	toilet area	toilet seat	N
20-Oct-99	swab	5	SR 8	main room	commode	N
20-Oct-99	swab	5			stand aid sling	P
20-Oct-99	swab	5	bay 4	toilet area	toilet handle	N
20-Oct-99	swab	5	bay 3	toilet area	toilet handle	N
20-Oct-99	swab	5	bay 2	toilet area	toilet seat	N
20-Oct-99	swab	5	bay 2	toilet area	toilet handle	N
20-Oct-99	swab	5	SR 8	main room	toilet door handle	N
20-Oct-99	contact plate	6	SR 10	toilet area	toilet floor	P
20-Oct-99	contact plate	6	bay 4	toilet area	toilet floor	N
20-Oct-99	contact plate	6	bay 1	toilet area	toilet floor	P
20-Oct-99	contact plate	6	SR 10	main room	floor	P
20-Oct-99	contact plate	6	SR 9	toilet area	toilet floor	P
20-Oct-99	contact plate	6	SR 9	main room	floor	P
20-Oct-99	contact plate	6	SR 6	toilet area	floor	N

Date of sampling	Sampling method	Ward	Area sampled			Result
20-Oct-99	contact plate	6	bay 3	toilet area	toilet seat	P
20-Oct-99	contact plate	6	bay 3	toilet area	floor	N
20-Oct-99	contact plate	6	bay 3	main bay	floor	P
20-Oct-99	contact plate	6	shower-room		shower seat	N
20-Oct-99	contact plate	6	shower-room		shower floor	N
20-Oct-99	contact plate	6	SR 6	main room	floor	P
20-Oct-99	contact plate	6	bathroom		bath	N
20-Oct-99	swab	6	bay 3	toilet area	toilet handle	N
20-Oct-99	swab	6	bay 1	toilet area	toilet handle	N
20-Oct-99	swab	6	SR 10	main room	toilet door handle	N
20-Oct-99	swab	6	SR 10	main room	toilet handle	N
20-Oct-99	swab	6	SR 9	main room	toilet door handle	N
20-Oct-99	swab	6	SR 9	main room	toilet seat	P
20-Oct-99	swab	6	SR 9	main room	toilet handle	N
20-Oct-99	swab	6	SR 6	main room	toilet seat	N
20-Oct-99	swab	6	bay 3	main bay	wash basin tap handle	N
20-Oct-99	swab	6	bay 3	toilet area	wash basin tap handle	P
20-Oct-99	swab	6	bay 3	toilet area	toilet handle	N
20-Oct-99	swab	6	sluice-room		bed pan disposal unit	N
20-Oct-99	swab	6	SR 6	main room	toilet handle	N
20-Oct-99	swab	6	bay 3	toilet area	toilet door handle	N
20-Oct-99	swab	6	SR 6	main room	toilet door handle	N
26-Oct-99	contact plate	5	bay 1	main bay	floor	N
26-Oct-99	contact plate	5	shower-room		shower floor	N

Date of sampling	Sampling method	Ward	Area sampled				Result
26-Oct-99	contact plate	5	shower-room			shower seat	N
26-Oct-99	contact plate	5	bathroom			bath	N
26-Oct-99	contact plate	5	bay	1	main bay	floor	P
26-Oct-99	contact plate	5	bay	1	toilet area	floor	N
26-Oct-99	contact plate	5	SR	8	toilet area	toilet floor	N
26-Oct-99	contact plate	5	bay	2	main bay	floor	N
26-Oct-99	contact plate	5	bay	2	toilet area	floor	N
26-Oct-99	contact plate	5	bay	3	main bay	floor	N
26-Oct-99	contact plate	5	bay	3	toilet area	floor	P
26-Oct-99	contact plate	5	bay	4	toilet area	floor	P
26-Oct-99	contact plate	5	SR	8	main room	floor	N
26-Oct-99	contact plate	5	bay	4	main bay	floor	N
26-Oct-99	swab	5	bay	2	toilet area	toilet door handle	N
26-Oct-99	swab	5	bay	4	toilet area	toilet door handle	N
26-Oct-99	swab	5	bay	4	toilet area	toilet handle	N
26-Oct-99	swab	5	bay	3	toilet area	toilet seat	N
26-Oct-99	swab	5	bay	3	toilet area	toilet handle	N
26-Oct-99	swab	5	bay	2	toilet area	toilet door handle	N
26-Oct-99	swab	5	SR	8	main room	toilet door handle	N
26-Oct-99	swab	5	SR	8	main room	commode	N
26-Oct-99	swab	5	SR	8	main room	toilet handle	N
26-Oct-99	swab	5				stand-aid sling	N
26-Oct-99	swab	5	bay	1	toilet area	toilet door handle	N
26-Oct-99	swab	5	bay	1	toilet area	toilet seat	N

Date of sampling	Sampling method	Ward	Area sampled				Result
26-Oct-99	swab	5	sluice-room			bed pan disposal unit	N
26-Oct-99	swab	5	bay	1	toilet area	toilet handles	N
26-Oct-99	contact plate	6	bathroom			bath	N
26-Oct-99	contact plate	6	shower-room			shower floor	N
26-Oct-99	contact plate	6	shower-room			shower seat	N
26-Oct-99	contact plate	6	bay	3	main bay	floor	N
26-Oct-99	contact plate	6	bay	1	main bay	floor	P
26-Oct-99	contact plate	6	bay	3	toilet area	toilet floor	P
26-Oct-99	contact plate	6	SR	5	toilet area	floor	P
26-Oct-99	contact plate	6	SR	10	toilet area	floor	P
26-Oct-99	contact plate	6	SR	10	main room	floor	P
26-Oct-99	contact plate	6	SR	9	toilet area	toilet floor	P
26-Oct-99	contact plate	6	SR	9	main room	floor	N
26-Oct-99	contact plate	6	SR	6	toilet area	toilet floor	P
26-Oct-99	contact plate	6	SR	6	toilet area	floor	P
26-Oct-99	contact plate	6	bay	3	toilet area	toilet seat	P
26-Oct-99	contact plate	6	SR	7	toilet area	floor	P
26-Oct-99	swab	6	sluice-room			bed pan disposal unit	N
26-Oct-99	swab	6	bay	3	toilet area	toilet handle	N
26-Oct-99	swab	6	bay	3	toilet area	toilet door handle	N
26-Oct-99	swab	6	bay	3	toilet area	toilet seat	P
26-Oct-99	swab	6	bay	3	toilet area	commode	N
26-Oct-99	swab	6	bay	3	main bay	wash basin tap handle	N
26-Oct-99	swab	6	SR	9	main room	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled				Result
26-Oct-99	swab	6	SR	6	main room	toilet seat	N
26-Oct-99	swab	6	SR	9	main room	zimmer frame	P
26-Oct-99	swab	6	SR	10	main room	toilet handle	N
26-Oct-99	swab	6	SR	5	main room	toilet handle	N
26-Oct-99	swab	6	SR	7	main room	toilet door handle	N
26-Oct-99	swab	6	SR	6	main room	toilet handle	N
26-Oct-99	swab	6	SR	9	main room	toilet seat	N
19-Nov-99	contact plate	5	bay	1	toilet area	floor	N
19-Nov-99	contact plate	5	bathroom			bath	N
19-Nov-99	contact plate	5	shower-room			shower seat	N
19-Nov-99	contact plate	5	shower-room			shower floor	N
19-Nov-99	contact plate	5	bathroom			bath	P
19-Nov-99	contact plate	5	bay	4	main bay	floor	N
19-Nov-99	contact plate	5	bay	4	toilet area	floor	P
19-Nov-99	contact plate	5	SR	7	main room	floor	N
19-Nov-99	contact plate	5	SR	7	main room	floor	N
19-Nov-99	contact plate	5	bay	3	main bay	floor	N
19-Nov-99	contact plate	5	bay	3	toilet area	floor	N
19-Nov-99	contact plate	5	bay	2	main bay	floor	N
19-Nov-99	contact plate	5	bay	1	main bay	floor	N
19-Nov-99	contact plate	5	bay	2	toilet area	floor	P
19-Nov-99	swab	5	bay	4	toilet area	toilet handle	N
19-Nov-99	swab	5	bay	3	toilet area	toilet seat	N
19-Nov-99	swab	5	bay	1	toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
19-Nov-99	swab	5	bay 2	toilet area	toilet seat	N
19-Nov-99	swab	5	bay 2	toilet area	toilet handle	N
19-Nov-99	swab	5	bay 3	toilet area	toilet handle	N
19-Nov-99	swab	5	SR 7	toilet area	toilet door handle	N
19-Nov-99	swab	5	SR 7	main room	commode	N
19-Nov-99	swab	5	SR 7	toilet area	toilet handle	N
19-Nov-99	swab	5	bay 4	toilet area	commode	N
19-Nov-99	swab	5	sluice-room		bed pan disposal unit	N
19-Nov-99	swab	5	bay 4	toilet area	toilet door handle	N
19-Nov-99	swab	5	bay 1	toilet area	toilet door handle	N
19-Nov-99	swab	5			stand-aid sling	N
19-Nov-99	contact plate	6	bathroom		bath	N
19-Nov-99	contact plate	6	bay 4	toilet area	toilet seat	N
19-Nov-99	contact plate	6	shower-room		shower floor	N
19-Nov-99	contact plate	6	SR 9	toilet area	floor	P
19-Nov-99	contact plate	6	shower-room		shower seat	P
19-Nov-99	contact plate	6	bay 1	main bay	floor	N
19-Nov-99	contact plate	6	bay 1	toilet area	floor	P
19-Nov-99	contact plate	6	bay 3	main bay	floor	P
19-Nov-99	contact plate	6	bay 3	toilet area	floor	P
19-Nov-99	contact plate	6	bay 3	toilet area	toilet seat	N
19-Nov-99	contact plate	6	SR 5	main room	floor	P
19-Nov-99	contact plate	6	SR 5	toilet area	floor	P
19-Nov-99	contact plate	6	SR 6	main room	floor	P

Date of sampling	Sampling method	Ward	Area sampled				Result
19-Nov-99	contact plate	6	SR	6	toilet area	floor	P
19-Nov-99	contact plate	6	SR	7	main room	floor	N
19-Nov-99	contact plate	6	SR	7	toilet area	floor	P
19-Nov-99	contact plate	6	SR	9	main room	floor	P
19-Nov-99	swab	6	SR	5	toilet area	toilet handle	P
19-Nov-99	swab	6	bay	4	toilet area	toilet seat	N
19-Nov-99	swab	6	SR	9	toilet area	toilet seat	N
19-Nov-99	swab	6	SR	6	toilet area	toilet seat	N
19-Nov-99	swab	6	SR	7	toilet area	toilet handle	P
19-Nov-99	swab	6	SR	5	toilet area	toilet door handle	N
19-Nov-99	swab	6	bay	3	toilet area	toilet door handle	N
19-Nov-99	swab	6	bay	3	toilet area	toilet seat	P
19-Nov-99	swab	6	bay	1	toilet area	toilet door handle	N
19-Nov-99	swab	6	bay	1	toilet area	toilet handle	N
19-Nov-99	swab	6	sluice-room			bed pan disposal unit	N
19-Nov-99	swab	6	SR	6	toilet area	toilet handle	N
19-Nov-99	swab	6	bay	3	toilet area	toilet handle	P
19-Nov-99	swab	6	bay	1	toilet area	toilet seat	N
25-Nov-99	contact plate	5	bay	2	toilet area	floor	N
25-Nov-99	contact plate	5	bay	2	main bay	floor	N
25-Nov-99	contact plate	5	bay	1	toilet area	floor	N
25-Nov-99	contact plate	5	bay	1	main bay	floor	N
25-Nov-99	contact plate	5	shower-room			shower floor	N
25-Nov-99	contact plate	5	shower-room			shower seat	N

Date of sampling	Sampling method	Ward	Area sampled			Result
25-Nov-99	contact plate	5	bathroom		bath	N
25-Nov-99	contact plate	5	bay 3	main bay	floor	P
25-Nov-99	contact plate	5	bathroom		bath	N
25-Nov-99	contact plate	5	bay 3	toilet area	toilet seat	N
25-Nov-99	contact plate	5	bay 4	main bay	floor	N
25-Nov-99	contact plate	5	bay 4	toilet area	floor	P
25-Nov-99	contact plate	5	SR 7	main room	floor	N
25-Nov-99	contact plate	5	SR 7	toilet area	floor	N
25-Nov-99	swab	5	bay 2	toilet area	toilet door handle	N
25-Nov-99	swab	5	bay 4	toilet area	Wash basin tap handle	N
25-Nov-99	swab	5	bay 4	toilet area	toilet door handle	N
25-Nov-99	swab	5	SR 7	toilet area	toilet handles	N
25-Nov-99	swab	5	bay 4	toilet area	toilet handle	N
25-Nov-99	swab	5	bay 1	toilet area	toilet door handle	N
25-Nov-99	swab	5	bay 1	toilet area	commode	N
25-Nov-99	swab	5	bay 1	toilet area	toilet handle	N
25-Nov-99	swab	5	sluice-room		bed pan disposal unit	N
25-Nov-99	swab	5	bay 2	toilet area	toilet seat	N
25-Nov-99	swab	5	bay 2	toilet area	toilet door handle	N
25-Nov-99	swab	5	bay 2	toilet area	toilet handle	N
25-Nov-99	swab	5	bay 3	toilet area	toilet seat	N
25-Nov-99	swab	5	bay 3	toilet area	toilet handle	N
25-Nov-99	contact plate	6	SR 6	toilet area	toilet floor	P
25-Nov-99	contact plate	6	SR 9	toilet area	toilet seat	N

Date of sampling	Sampling method	Ward	Area sampled				Result
25-Nov-99	contact plate	6	bay	1	main bay	floor	N
25-Nov-99	contact plate	6	bay	4	main bay	floor	N
25-Nov-99	contact plate	6	bay	4	toilet area	floor	P
25-Nov-99	contact plate	6	SR	5	main room	floor	P
25-Nov-99	contact plate	6	SR	5	toilet area	toilet	N
25-Nov-99	contact plate	6	SR	6	main room	floor	P
25-Nov-99	contact plate	6	SR	9	main room	floor	P
25-Nov-99	contact plate	6	shower-room			shower floor	N
25-Nov-99	contact plate	6	bathroom			bath	N
25-Nov-99	contact plate	6	shower-room			shower seat	N
25-Nov-99	contact plate	6	bay	3	toilet area	toilet seat	P
25-Nov-99	contact plate	6	bay	3	main bay	floor	N
25-Nov-99	contact plate	6	bay	3	toilet area	floor	P
25-Nov-99	swab	6	bay	3	toilet area	toilet seat	N
25-Nov-99	swab	6	bay	3	toilet area	toilet handles	N
25-Nov-99	swab	6	bay	3	toilet area	toilet door handle	N
25-Nov-99	swab	6	bay	3	main bay	Wash basin tap handle	N
25-Nov-99	swab	6	bay	4	toilet area	toilet handles	N
25-Nov-99	swab	6	bay	4	toilet area	toilet seat	N
25-Nov-99	swab	6	bay	4	toilet area	toilet door handle	N
25-Nov-99	swab	6	SR	5	toilet area	toilet handle	N
25-Nov-99	swab	6	SR	5	toilet area	toilet door handle	N
25-Nov-99	swab	6	SR	6	toilet area	toilet handle	N
25-Nov-99	swab	6	SR	6	main room	Commode handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
25-Nov-99	swab	6	SR 9 toilet area	toilet handles		N
25-Nov-99	swab	6	SR 9 toilet area	toilet seat		N
25-Nov-99	swab	6	sluice-room	bed pan disposal unit		N
02-Dec-99	contact plate	5	shower-room	shower floor		N
02-Dec-99	contact plate	5	sluice-room	commode		P
02-Dec-99	contact plate	5	bay 1 main bay	floor		N
02-Dec-99	contact plate	5	bathroom	bath		N
02-Dec-99	contact plate	5	bathroom	bath		N
02-Dec-99	contact plate	5	shower-room	shower seat		P
02-Dec-99	contact plate	5	bay 1 toilet area	toilet floor		P
02-Dec-99	contact plate	5	bay 2 main bay	floor		P
02-Dec-99	contact plate	5	bay 2 toilet area	toilet floor		N
02-Dec-99	contact plate	5	bay 3 main bay	floor		N
02-Dec-99	contact plate	5	bay 3 toilet area	toilet floor		N
02-Dec-99	contact plate	5	bay 4 main bay	floor		N
02-Dec-99	contact plate	5	SR 9 toilet area	toilet floor		P
02-Dec-99	contact plate	5	bay 4 toilet area	toilet floor		N
02-Dec-99	swab	5	bay 3 toilet area	toilet handle		N
02-Dec-99	swab	5	bay 2 toilet area	toilet door handle		N
02-Dec-99	swab	5	bay 3 toilet area	toilet seat		N
02-Dec-99	swab	5	bay 2 toilet area	toilet handle		N
02-Dec-99	swab	5	bay 1 toilet area	toilet door handle		N
02-Dec-99	swab	5	bay 1 toilet area	toilet seat		N
02-Dec-99	swab	5	bay 1 toilet area	toilet handle		N

Date of sampling	Sampling method	Ward	Area sampled				Result
02-Dec-99	swab	5	sluice-room			commode handle	N
02-Dec-99	swab	5	sluice-room			bed pan disposal unit	N
02-Dec-99	swab	5	bay	4	toilet area	toilet handle	P
02-Dec-99	swab	5	bay	2	toilet area	toilet seat	N
02-Dec-99	swab	5	bay	4	toilet area	toilet door handle	N
02-Dec-99	swab	5	SR	9	toilet area	toilet seat	P
02-Dec-99	swab	5	SR	9	toilet area	toilet handle	P
02-Dec-99	contact plate	6	bay	4	toilet area	floor	P
02-Dec-99	contact plate	6	bay	1	toilet area	floor	P
02-Dec-99	contact plate	6	SR	9	toilet area	toilet seat	N
02-Dec-99	contact plate	6	SR	9	toilet area	toilet floor	P
02-Dec-99	contact plate	6	SR	9	main room	floor	P
02-Dec-99	contact plate	6	SR	6	main room	Commode	N
02-Dec-99	contact plate	6	SR	6	toilet area	toilet floor	N
02-Dec-99	contact plate	6	sluice-room			commode	N
02-Dec-99	contact plate	6	bay	3	toilet area	toilet seat	N
02-Dec-99	contact plate	6	bay	3	toilet area	toilet floor	N
02-Dec-99	contact plate	6	SR	6	main room	floor	N
02-Dec-99	contact plate	6	bay	3	main bay	floor	N
02-Dec-99	contact plate	6	shower-room			shower floor	P
02-Dec-99	contact plate	6	shower-room			shower seat	N
02-Dec-99	contact plate	6	bathroom			bath	N
02-Dec-99	swab	6	SR	9	toilet area	toilet handle	N
02-Dec-99	swab	6	bay	3	main bay	Wash basin tap handle	N

Date of sampling	Sampling method	Ward	Area sampled				Result
02-Dec-99	swab	6	bay	4	toilet area	toilet seat	N
02-Dec-99	swab	6	bay	1	toilet area	toilet handle	N
02-Dec-99	swab	6	SR	9	toilet area	toilet door handle	N
02-Dec-99	swab	6	SR	9	toilet area	toilet seat	N
02-Dec-99	swab	6	SR	6	toilet area	toilet door handle	N
02-Dec-99	swab	6	SR	6	toilet area	toilet seat	N
02-Dec-99	swab	6	SR	6	toilet area	toilet handle	N
02-Dec-99	swab	6	bay	3	toilet area	toilet door handle	N
02-Dec-99	swab	6	bay	3	toilet area	toilet seat	N
02-Dec-99	swab	6	bay	3	toilet area	toilet handle	N
02-Dec-99	swab	6	sluice-room			commode handle	N
02-Dec-99	swab	6	sluice-room			bed pan disposal unit	N
21-Jan-00	contact plate	5	kitchen			floor	N
21-Jan-00	contact plate	5	bathroom			ambulift	N
21-Jan-00	contact plate	5	sluice-room			commode	P
21-Jan-00	contact plate	5	bathroom			floor	N
21-Jan-00	contact plate	5	bathroom			floor	P
21-Jan-00	contact plate	5	bay	3	main bay	windowsill	N
21-Jan-00	contact plate	5	bay	3	toilet area	chair	N
21-Jan-00	contact plate	5	bay	4	main bay	bedcover	N
21-Jan-00	contact plate	5	bay	2	main bay	armchair	N
21-Jan-00	contact plate	5	bay	2	toilet area	toilet seat	N
21-Jan-00	contact plate	5	bay	1	toilet area	floor	P
21-Jan-00	contact plate	5	SR	10	main room	locker-top	P

Date of sampling	Sampling method	Ward	Area sampled		Result
21-Jan-00	contact plate	5	sluice-room	bed pan disposal unit	P
21-Jan-00	contact plate	5	bay 1 main bay	floor	N
21-Jan-00	swab	5	bay 1 main bay	zimmer frame	N
21-Jan-00	swab	5	bay 3 main bay	wash basin tap handle	N
21-Jan-00	swab	5	bay 4 main bay	monkey handle	N
21-Jan-00	swab	5		stand-aid sling	N
21-Jan-00	swab	5	bathroom	ambulift handle	N
21-Jan-00	swab	5	bathroom	toilet handle	N
21-Jan-00	swab	5	bay 3 main bay	armchair	N
21-Jan-00	swab	5	bathroom	door handle	N
21-Jan-00	swab	5	bathroom	shower curtain	N
21-Jan-00	swab	5	bay 1 toilet area	toilet handles	N
21-Jan-00	swab	5	sluice-room	commode handle	N
21-Jan-00	swab	5	bay 2 toilet area	wash basin tap handle	N
21-Jan-00	swab	5	SR 10 toilet area	toilet door handle	N
21-Jan-00	swab	5	bay 2 toilet area	wash basin tap handle	N
21-Jan-00	contact plate	6	bay 1 toilet area	wash basin tap handle	N
21-Jan-00	contact plate	6	bay 3 main bay	floor	N
21-Jan-00	contact plate	6	SR 8 main room	floor	N
21-Jan-00	contact plate	6	bay 4 main bay	floor	N
21-Jan-00	contact plate	6	bay 3 toilet area	floor	N
21-Jan-00	contact plate	6	bay 2 main bay	floor	N
21-Jan-00	contact plate	6	bay 2 toilet area	floor	N
21-Jan-00	contact plate	6	bay 1 toilet area	toilet seat	N

Date of sampling	Sampling method	Ward	Area sampled				Result
21-Jan-00	contact plate	6	sluice-room			bed pan disposal unit	P
21-Jan-00	contact plate	6	sluice-room			commode	P
21-Jan-00	contact plate	6	bathroom			ambulift	N
21-Jan-00	contact plate	6	bathroom			floor	P
21-Jan-00	contact plate	6	kitchen			floor	P
21-Jan-00	contact plate	6	bay	1	toilet area	floor	P
21-Jan-00	swab	6	bay	2	toilet area	wash basin tap handle	N
21-Jan-00	swab	6	SR	8	main room	wash basin tap handle	N
21-Jan-00	swab	6	bathroom			raised seat handle	N
21-Jan-00	swab	6	bay	1	toilet area	wash basin tap handle	N
21-Jan-00	swab	6	bay	2	main bay	wash basin tap handle	N
21-Jan-00	swab	6	bay	2	toilet area	toilet handle	N
21-Jan-00	swab	6	bay	4	toilet area	wash basin tap handle	N
21-Jan-00	swab	6				stand-aid sling	N
21-Jan-00	swab	6	SR	8	main room	door handle	N
21-Jan-00	swab	6			main ward	door handle	N
21-Jan-00	swab	6	shower-room			shower door handle	N
21-Jan-00	swab	6	bathroom			ambulift handle	N
21-Jan-00	swab	6	sluice-room			commode handle	N
21-Jan-00	swab	6	bay	3	toilet area	toilet seat	N
28-Jan-00	contact plate	6	bay	3	main bay	chair	N
28-Jan-00	contact plate	6	bay	3	main bay	clinical waste holder	N
28-Jan-00	contact plate	6	SR	8	toilet area	paper towel dispenser	P
28-Jan-00	contact plate	6	bay	3	main bay	armchair	N

Date of sampling	Sampling method	Ward	Area sampled				Result
28-Jan-00	contact plate	6	bay	3	toilet area	towel dispenser	P
28-Jan-00	contact plate	6	bay	3	toilet area	cistern	P
28-Jan-00	contact plate	6	bay	3	main bay	patient tray	N
28-Jan-00	contact plate	6	bay	3	main bay	locker	N
28-Jan-00	contact plate	6	SR	8	main room	chair	P
28-Jan-00	contact plate	6	SR	8	toilet area	cistern	P
28-Jan-00	contact plate	6	bay	3	main bay	window sill	P
28-Jan-00	contact plate	6	bathroom			floor	N
28-Jan-00	contact plate	6	SR	8	main room	window sill	N
28-Jan-00	contact plate	6	sluic			shelf	P
28-Jan-00	contact plate	6	SR	8	main room	wash bowl	P
28-Jan-00	contact plate	6	kitchen			paper towel dispenser	N
28-Jan-00	contact plate	6	sluice-room			bed pan disposal unit	N
28-Jan-00	contact plate	6	sluice-room			shelf	N
28-Jan-00	contact plate	6	sluice-room			cupboard	P
28-Jan-00	contact plate	6	sluice-room			shelf	N
28-Jan-00	contact plate	6	sluice-room			commode	P
28-Jan-00	contact plate	6	bathroom			chair	N
28-Jan-00	contact plate	6	sluice-room			floor	P
28-Jan-00	contact plate	6	bathroom			bath	P
28-Jan-00	contact plate	6	kitchen			cupboard	N
28-Jan-00	contact plate	6	bathroom			ambulift	P
28-Jan-00	contact plate	6	bathroom			toilet seat	N
28-Jan-00	contact plate	6	sluice-room			commode	N

Date of sampling	Sampling method	Ward	Area sampled		Result
28-Jan-00	contact plate	6	bathroom	Shelf	P
28-Jan-00	contact plate	6	sluice-room	radiator	N
28-Jan-00	swab	6	sluice-room	bed pan disposal unit	N
28-Jan-00	swab	6	sluice-room	commode	N
28-Jan-00	swab	6	sluice-room	commode	N
28-Jan-00	swab	6	sluice-room	wash basin tap handle	N
28-Jan-00	swab	6	sluice-room	mop	N
28-Jan-00	swab	6	sluice-room	wash basin tap handle	N
28-Jan-00	swab	6	sluice-room	mop	N
28-Jan-00	swab	6	SR 8 toilet area	toilet support handle	N
28-Jan-00	swab	6	bay 3 main bay	door handle	N
28-Jan-00	swab	6	bay 3 main bay	locker	N
28-Jan-00	swab	6	bay 3 main bay	zimmer frame	N
28-Jan-00	swab	6	bay 3 toilet area	curtain	N
28-Jan-00	swab	6	bay 3 toilet area	shelf	N
28-Jan-00	swab	6	bay 3 main bay	patient tray	N
28-Jan-00	swab	6	bay 3 main bay	bed	N
28-Jan-00	swab	6	bay 3 main bay	bed	P
28-Jan-00	swab	6	SR 8 main room	door handle	P
28-Jan-00	swab	6	SR 8 main room	nurse trolley	N
28-Jan-00	swab	6	bathroom	tap	N
28-Jan-00	swab	6	SR 8 main room	bed	P
28-Jan-00	swab	6	kitchen	tap handle	N
28-Jan-00	swab	6	kitchen	tap handle	N

Date of sampling	Sampling method	Ward	Area sampled		Result
28-Jan-00	swab	6	bathroom	shower curtain	N
28-Jan-00	swab	6	bathroom	wash basin tap handle	N
28-Jan-00	swab	6	bathroom	toilet seat	N
28-Jan-00	swab	6	bathroom	toilet handle	N
28-Jan-00	swab	6	bathroom	ambulift	N
28-Jan-00	swab	6	SR 8 main room	patient trolley	N
04-Feb-00	contact plate	5	bay 1 main bay	armchair	N
04-Feb-00	contact plate	5	bay 1 toilet area	shelf	N
04-Feb-00	contact plate	5	SR 10 toilet area	floor	N
04-Feb-00	contact plate	5	SR 10 main room	paper towel dispenser	N
04-Feb-00	contact plate	5	SR 10 toilet area	cistern	N
04-Feb-00	contact plate	5	SR 10 main room	patient trolley	N
04-Feb-00	contact plate	5	SR 10 main room	window sill	N
04-Feb-00	contact plate	5	sluice-room	cupboard	N
04-Feb-00	contact plate	5	bay 1 main bay	windowsill	N
04-Feb-00	contact plate	5	bay 1 main bay	chair	N
04-Feb-00	contact plate	5	SR 10 main room	foot stool	N
04-Feb-00	contact plate	5	sluice-room	radiator	N
04-Feb-00	contact plate	5	sluice-room	commode	N
04-Feb-00	contact plate	5	bathroom	cupboard	N
04-Feb-00	contact plate	5	sluice-room	floor	N
04-Feb-00	contact plate	5	bathroom	bath	N
04-Feb-00	contact plate	5	bathroom	floor	N
04-Feb-00	contact plate	5	bathroom	chair	N

Date of sampling	Sampling method	Ward	Area sampled		Result
04-Feb-00	contact plate	5	bathroom	shower curtain	N
04-Feb-00	contact plate	5	bay 1 main bay	patient-tray	N
04-Feb-00	contact plate	5	bathroom	cistern	N
04-Feb-00	contact plate	5	bathroom	scales	N
04-Feb-00	contact plate	5	bathroom	ambulift	N
04-Feb-00	contact plate	5	bathroom	radiator	N
04-Feb-00	contact plate	5	bathroom	shelf	N
04-Feb-00	contact plate	5	bay 1 main bay	locker	N
04-Feb-00	contact plate	5	bay 1 main bay	patient-tray	N
04-Feb-00	contact plate	5	sluice-room	commode	N
04-Feb-00	swab	5	sluice-room	wash basin tap handle	N
04-Feb-00	swab	5	SR 10 main room	monkey pole-handle	N
04-Feb-00	swab	5	sluice-room	mop	N
04-Feb-00	swab	5	sluice-room	mop	N
04-Feb-00	swab	5	sluice-room	wash basin tap handle	N
04-Feb-00	swab	5	SR 10 toilet area	toilet handrail	N
04-Feb-00	swab	5	SR 10 main room	wash basin tap handle	N
04-Feb-00	swab	5	sluice-room	commode	N
04-Feb-00	swab	5	bathroom	tap	N
04-Feb-00	swab	5	sluice-room	commode	N
04-Feb-00	swab	5	SR 10 main room	bed	N
04-Feb-00	swab	5	bay 1 toilet area	toilet-handrail	N
04-Feb-00	swab	5	bay 1 toilet area	toilet-handrail	N
04-Feb-00	swab	5	bay 1 main bay	equipment tray	N

Date of sampling	Sampling method	Ward	Area sampled		Result
04-Feb-00	swab	5	bay 1 main bay	wash basin tap handle	N
04-Feb-00	swab	5	bay 1 main bay	lamp	N
04-Feb-00	swab	5	bathroom	tap handle	N
04-Feb-00	swab	5	bathroom	ambulift	N
04-Feb-00	swab	5	sluice-room	commode	N
04-Feb-00	swab	5	bathroom	tap handle	N
04-Feb-00	swab	5	bathroom	tap	N
04-Feb-00	swab	5	bathroom	door handle	N
04-Feb-00	swab	5	bathroom	bath	N
04-Feb-00	swab	5	bathroom	bath	N
04-Feb-00	swab	5	bathroom	toilet-handrail	N
04-Feb-00	swab	5	bathroom	wash basin tap handle	N
04-Feb-00	swab	5	bathroom	scales	N
04-Feb-00	swab	5	sluice-room	shelf	N
15-Feb-00	contact plate	6	SR 5 main room	window sill	N
15-Feb-00	contact plate	6	SR 5 main room	floor	N
15-Feb-00	contact plate	6	SR 5 toilet area	floor	P
15-Feb-00	contact plate	6	SR 8 main room	window sill	P
15-Feb-00	contact plate	6	sluice-room	cupboard	N
15-Feb-00	contact plate	6	bathroom	shelf	N
15-Feb-00	contact plate	6	bay 1 toilet area	cistern	N
15-Feb-00	contact plate	6	bay 1 main bay	chair	N
15-Feb-00	contact plate	6	bay 1 main bay	patient trolley	N
15-Feb-00	contact plate	6	bay 1 main bay	shelf	N

Date of sampling	Sampling method	Ward	Area sampled			Result
15-Feb-00	contact plate	6	bathroom		ambulift	P
15-Feb-00	contact plate	6	kitchen		cupboard	N
15-Feb-00	contact plate	6	bathroom		chair	N
15-Feb-00	contact plate	6	sluice-room		shelf	P
15-Feb-00	contact plate	6	sluice-room		radiator	N
15-Feb-00	contact plate	6	sluice-room		wash basin	N
15-Feb-00	contact plate	6	bathroom		ambulift	N
15-Feb-00	contact plate	6	bathroom		toilet seat	N
15-Feb-00	contact plate	6	bay 1	main bay	locker	N
15-Feb-00	contact plate	6	bay 3	main bay	armchair	N
15-Feb-00	contact plate	6	SR 8	main room	seat	P
15-Feb-00	contact plate	6	SR 8	toilet area	cistern	P
15-Feb-00	contact plate	6	SR 8	main room	window sill	N
15-Feb-00	contact plate	6	bay 1	toilet area	toilet waste pipe	N
15-Feb-00	contact plate	6	bay 3	main bay	clinical waste holder	N
15-Feb-00	contact plate	6	kitchen		paper towel holder	P
15-Feb-00	contact plate	6	bay 3	main bay	seat	P
15-Feb-00	contact plate	6	bay 3	toilet area	shelf	N
15-Feb-00	contact plate	6	bay 3	main bay	patient trolley	N
15-Feb-00	contact plate	6	bay 3	main bay	locker	N
15-Feb-00	contact plate	6	kitchen		floor	N
15-Feb-00	contact plate	6	SR 8	main room	locker	P
15-Feb-00	swab	6	bathroom		tap	N
15-Feb-00	swab	6	kitchen		tap handle	N

Date of sampling	Sampling method	Ward	Area sampled		Result
15-Feb-00	swab	6	kitchen	door handle	N
15-Feb-00	swab	6	kitchen	tap	N
15-Feb-00	swab	6	bay 1 toilet area	trays	N
15-Feb-00	swab	6	bay 1 main bay	wash basin tap handle	N
15-Feb-00	swab	6	bay 1 main bay	bed	N
15-Feb-00	swab	6	bay 1 main bay	floor	N
15-Feb-00	swab	6	bay 1 main bay	armchair	N
15-Feb-00	swab	6	SR 8 main room	armchair	N
15-Feb-00	swab	6	bay 3 main bay	window sill	N
15-Feb-00	swab	6	bathroom	door handle	N
15-Feb-00	swab	6	bay 3 main bay	locker	N
15-Feb-00	swab	6	bay 3 main bay	floor	N
15-Feb-00	swab	6	bay 3 toilet area	toilet handrail	N
15-Feb-00	swab	6	bay 3 main bay	wash basin tap handle	N
15-Feb-00	swab	6	bay 3 main bay	catheter bag holder	N
15-Feb-00	swab	6	SR 8 main room	bed	N
15-Feb-00	swab	6	SR 8 main room	shelf	N
15-Feb-00	swab	6	bathroom	ambulift	N
15-Feb-00	swab	6	bathroom	seat	N
15-Feb-00	swab	6	SR 8 main room	wash basin tap handle	N
15-Feb-00	swab	6	bathroom	wash basin tap handle	N
15-Feb-00	swab	6	bay 1 main bay	lamp	N
15-Feb-00	swab	6	sluice-room	commode	N
15-Feb-00	swab	6	sluice-room	mop	N

Date of sampling	Sampling method	Ward	Area sampled		Result
15-Feb-00	swab	6	sluice-room	mop	N
15-Feb-00	swab	6	sluice-room	tap handle	N
20-Mar-00	contact plate	5	bay 2 main bay	floor	N
20-Mar-00	contact plate	5	bay 2 main bay	locker	N
20-Mar-00	contact plate	5	bay 2 toilet area	floor	N
20-Mar-00	contact plate	5	bathroom	ambulift	N
20-Mar-00	contact plate	5	bay 1 wash area	seat	P
20-Mar-00	contact plate	5	bay 1 toilet area	toilet seat	P
20-Mar-00	contact plate	5	bay 1 main bay	patient table top	N
20-Mar-00	contact plate	5	bay 1 main bay	floor	P
20-Mar-00	contact plate	5	bathroom	scales	N
20-Mar-00	contact plate	5	shower-room	shower seat	N
20-Mar-00	contact plate	5	shower-room	floor	P
20-Mar-00	contact plate	5	bathroom	floor	N
20-Mar-00	contact plate	5	sluice-room	commode	N
20-Mar-00	contact plate	5	bay 2 wash area	seat	N
20-Mar-00	contact plate	5	bathroom	chair	P
20-Mar-00	contact plate	5	bay 3 main bay	floor	N
20-Mar-00	contact plate	5	sluice-room	floor	P
20-Mar-00	contact plate	5	bay 4 wash area	seat	N
20-Mar-00	contact plate	5	bay 3 toilet area	cistern	N
20-Mar-00	contact plate	5	bay 3 wash area	shelf	N
20-Mar-00	contact plate	5	bay 4 main bay	floor	P
20-Mar-00	contact plate	5	bay 4 toilet area	floor	N

Date of sampling	Sampling method	Ward	Area sampled				Result
20-Mar-00	contact plate	5	SR	7	main room	locker	N
20-Mar-00	contact plate	5	SR	7	main room	window sill	N
20-Mar-00	contact plate	5	SR	7	toilet area	cistern	N
20-Mar-00	contact plate	5	SR	8	main room	floor	P
20-Mar-00	contact plate	5	bay	4	main bay	patient table top	N
20-Mar-00	contact plate	5	SR	8	main room	armchair	P
20-Mar-00	contact plate	5	SR	8	toilet area	floor	P
20-Mar-00	contact plate	5	bay	3	main bay	locker	N
20-Mar-00	swab	5	bay	4	main bay	bed	N
20-Mar-00	swab	5	bay	1	main bay	monkey pole	P
20-Mar-00	swab	5	bay	1	toilet area	wash basin tap handle	N
20-Mar-00	swab	5	bay	1	wash area	equipment tray	N
20-Mar-00	swab	5	bay	2	main bay	armchair	N
20-Mar-00	swab	5	bay	2	main bay	wash basin tap handle	N
20-Mar-00	swab	5	bay	2	toilet area	toilet handle	N
20-Mar-00	swab	5	bay	2	main bay	paper towel dispenser	N
20-Mar-00	swab	5	bay	3	main bay	bed	N
20-Mar-00	swab	5	bay	1	main bay	bed	N
20-Mar-00	swab	5	bay	3	toilet area	toilet support handle	N
20-Mar-00	swab	5	bay	3	wash area	wash basin tap handle	N
20-Mar-00	swab	5	bay	4	toilet area	toilet door handle	N
20-Mar-00	swab	5	bay	4	main bay	wash basin tap handle	N
20-Mar-00	swab	5	SR	8	main room	patient table top	N
20-Mar-00	swab	5	SR	8	toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled				Result
20-Mar-00	swab	5	SR	7	main room	equipment trolley	N
20-Mar-00	swab	5	SR	7	main room	door handle	N
20-Mar-00	swab	5	bay	3	main bay	lamp	N
20-Mar-00	swab	5	bay	4	main bay	bed	N
20-Mar-00	swab	5	kitchen			mop	N
20-Mar-00	swab	5	bathroom			toilet handle	N
20-Mar-00	swab	5	bathroom			scales	N
20-Mar-00	swab	5	shower-room			shower seat	N
20-Mar-00	swab	5	bathroom			wash basin tap handle	N
20-Mar-00	swab	5	sluice-room			commode	N
20-Mar-00	swab	5	sluice-room			bed pan disposal unit	N
14-Jul-00	contact plate	5	bathroom			ambulift	N
14-Jul-00	contact plate	5	shower-room			shower floor	N
14-Jul-00	contact plate	5	bathroom			floor	N
14-Jul-00	contact plate	5	bay	1	main bay	bed	N
14-Jul-00	contact plate	5	bay	1	toilet area	toilet seat	N
14-Jul-00	contact plate	5	bay	2	main bay	seat	N
14-Jul-00	contact plate	5	bay	2	toilet area	floor	N
14-Jul-00	contact plate	5	bay	3	main bay	floor	N
14-Jul-00	contact plate	5	bay	3	toilet area	floor	N
14-Jul-00	contact plate	5	bay	4	main bay	bed	N
14-Jul-00	contact plate	5	bay	4	main bay	patient trolley	N
14-Jul-00	contact plate	5	SR	8	main room	window sill	N
14-Jul-00	contact plate	5	sluice-room			commode	N

Date of sampling	Sampling method	Ward	Area sampled				Result
14-Jul-00	contact plate	5	SR	8	main room	toilet floor	N
14-Jul-00	swab	5	SR	8	toilet area	toilet handle	N
14-Jul-00	swab	5	bay	4	main bay	lamp	N
14-Jul-00	swab	5	bay	3	toilet area	toilet seat	N
14-Jul-00	swab	5	bay	3	main bay	locker	N
14-Jul-00	swab	5	bay	2	main bay	bed	N
14-Jul-00	swab	5	bay	2	main bay	wash basin tap handle	N
14-Jul-00	swab	5	bathroom			chair	N
14-Jul-00	swab	5	bay	1	main bay	bed	N
14-Jul-00	swab	5	bathroom			shelf	N
14-Jul-00	swab	5	sluice-room			bed pan disposal unit	N
14-Jul-00	swab	5	bay	1	toilet area	wash basin tap handle	N
14-Jul-00	swab	5	bay	4	toilet area	toilet door handle	N
14-Jul-00	swab	5	SR	8	main room	wardrobe	N
14-Jul-00	swab	5	sluice-room			mop	N
14-Jul-00	contact plate	6	SR	9	toilet area	floor	N
14-Jul-00	contact plate	6	SR	9	main room	floor	N
14-Jul-00	contact plate	6	bay	2	main bay	locker	N
14-Jul-00	contact plate	6	bay	4	main bay	floor	N
14-Jul-00	contact plate	6	bay	4	toilet area	seat	N
14-Jul-00	contact plate	6	bay	2	main bay	floor	N
14-Jul-00	contact plate	6	bay	3	toilet area	floor	N
14-Jul-00	contact plate	6	bay	1	toilet area	toilet seat	P
14-Jul-00	contact plate	6	bay	1	main bay	bed	N

Date of sampling	Sampling method	Ward	Area sampled		Result
14-Jul-00	contact plate	6	bathroom	floor	N
14-Jul-00	contact plate	6	bathroom	bath	N
14-Jul-00	contact plate	6	shower-room	floor	N
14-Jul-00	contact plate	6	shower-room	shower seat	N
14-Jul-00	contact plate	6	sluice-room	commode	N
14-Jul-00	contact plate	6	bay 3 main bay	locker	N
14-Jul-00	swab	6	SR 9 toilet area	commode	N
14-Jul-00	swab	6	sluice-room	bed pan disposal unit	N
14-Jul-00	swab	6	bathroom	ambulift	N
14-Jul-00	swab	6	bathroom	toilet handle	N
14-Jul-00	swab	6	bay 1 main bay	bed	N
14-Jul-00	swab	6	bay 2 main bay	wash basin tap handle	N
14-Jul-00	swab	6	bay 1 toilet area	toilet handle	N
14-Jul-00	swab	6	bay 2 toilet area	toilet seat	N
14-Jul-00	swab	6	bay 3 main bay	patient trolley	N
14-Jul-00	swab	6	bay 3 toilet area	toilet door handle	N
14-Jul-00	swab	6	bay 4 main bay	wash basin tap handle	N
14-Jul-00	swab	6	SR 9 main room	locker	N
14-Jul-00	swab	6	SR 9 toilet area	wash basin tap handle	N
14-Jul-00	swab	6	bay 4 toilet area	wash basin tap handle	N
03-Aug-00	contact plate	5	bay 2 toilet area	toilet seat	P
03-Aug-00	contact plate	5	SR 7 main room	floor	N
03-Aug-00	contact plate	5	sluice-room	commode	P
03-Aug-00	contact plate	5	sluice-room	floor	P

Date of sampling	Sampling method	Ward	Area sampled		Result
03-Aug-00	contact plate	5	bathroom	ambulift	N
03-Aug-00	contact plate	5	bathroom	floor	N
03-Aug-00	contact plate	5	shower-room	floor	N
03-Aug-00	contact plate	5	bay 1 main bay	floor	P
03-Aug-00	contact plate	5	bay 1 main bay	locker	N
03-Aug-00	contact plate	5	bay 2 toilet area	floor	P
03-Aug-00	contact plate	5	bay 3 toilet area	floor	N
03-Aug-00	contact plate	5	bay 3 main bay	locker	N
03-Aug-00	contact plate	5	bay 4 toilet area	floor	N
03-Aug-00	contact plate	5	bay 4 main bay	floor	N
03-Aug-00	swab	5	bay 3 main bay	monkey pole	N
03-Aug-00	swab	5	bay 2 main bay	door handle	N
03-Aug-00	swab	5	bay 2 main bay	window sill	N
03-Aug-00	swab	5	bay 1 main bay	wash basin tap handle	N
03-Aug-00	swab	5	bay 1 main bay	bed	N
03-Aug-00	swab	5	shower-room	shower seat	N
03-Aug-00	swab	5	bay 3 toilet area	toilet door handle	N
03-Aug-00	swab	5	bathroom	tap handle	N
03-Aug-00	swab	5	sluice-room	commode	N
03-Aug-00	swab	5	bathroom	ambulift	N
03-Aug-00	swab	5	bay 4 wash area	wash basin tap handle	N
03-Aug-00	swab	5	bay 4 toilet area	toilet door handle	N
03-Aug-00	swab	5	SR 7 main room	wash basin tap handle	N
03-Aug-00	swab	5	sluice-room	bed pan disposal unit	N

Date of sampling	Sampling method	Ward	Area sampled		Result
03-Aug-00	contact plate	6	bay 4 main bay	floor	N
03-Aug-00	contact plate	6	sluice-room	floor	P
03-Aug-00	contact plate	6	sluice-room	commode	N
03-Aug-00	contact plate	6	bathroom	bed pan disposal unit	N
03-Aug-00	contact plate	6	shower-room	shower seat	N
03-Aug-00	contact plate	6	bay 1 main bay	floor	P
03-Aug-00	contact plate	6	bay 1 main bay	locker	N
03-Aug-00	contact plate	6	bay 2 main bay	floor	N
03-Aug-00	contact plate	6	bay 2 toilet area	floor	N
03-Aug-00	contact plate	6	bay 3 main bay	floor	N
03-Aug-00	swab	6	shower-room	shower handle	N
03-Aug-00	swab	6	sluice-room	bed pan disposal unit	N
03-Aug-00	swab	6	bathroom	toilet handle	N
03-Aug-00	swab	6	bay 2 toilet area	wash basin tap handle	N
03-Aug-00	swab	6	bay 1 toilet area	toilet handle	N
03-Aug-00	swab	6		maxi hoist sling	N
03-Aug-00	swab	6	bay 1 toilet area	seat	N
03-Aug-00	swab	6	sluice-room	commode	N
03-Aug-00	swab	6	bay 2 main bay	wash basin tap handle	N
03-Aug-00	swab	6	bay 4 toilet area	seat	N
03-Aug-00	swab	6		stand aid hoist	N
03-Aug-00	swab	6	bay 3 main bay	armchair	N
03-Aug-00	swab	6	bay 4 main bay	locker	N
03-Aug-00	swab	6		stand aid hoist	N

Date of sampling	Sampling method	Ward	Area sampled		Result
08-Sep-00	contact plate	5	bay 1 main bay	floor	N
08-Sep-00	contact plate	5	sluice-room	floor	N
08-Sep-00	contact plate	5	sluice-room	commode	P
08-Sep-00	contact plate	5	bathroom	bath	N
08-Sep-00	contact plate	5	bathroom	shower screen	N
08-Sep-00	contact plate	5	shower-room	shower seat	N
08-Sep-00	contact plate	5	bay 1 toilet area	floor	N
08-Sep-00	contact plate	5	bay 2 main bay	floor	N
08-Sep-00	contact plate	5	bay 2 toilet area	floor	N
08-Sep-00	contact plate	5	bay 3 main bay	floor	N
08-Sep-00	contact plate	5	bay 3 toilet area	toilet seat	N
08-Sep-00	contact plate	5	bay 4 main bay	floor	N
08-Sep-00	contact plate	5	bay 4 main bay	locker	N
08-Sep-00	contact plate	5	shower-room	shower floor	N
08-Sep-00	swab	5	bathroom	ambulift	N
08-Sep-00	swab	5	bathroom	toilet handle	N
08-Sep-00	swab	5	shower-room	shower seat	N
08-Sep-00	swab	5	shower-room	shower door handle	N
08-Sep-00	swab	5	bay 1 main bay	wash basin tap handle	N
08-Sep-00	swab	5	bay 1 toilet area	toilet handle	N
08-Sep-00	swab	5	bay 2 toilet area	toilet seat	N
08-Sep-00	swab	5	bay 2 toilet area	toilet door handle	N
08-Sep-00	swab	5	bay 3 toilet area	toilet seat	N
08-Sep-00	swab	5	bay 4 toilet area	toilet seat	N

Date of sampling	Sampling method	Ward	Area sampled		Result
08-Sep-00	swab	5	sluice-room		N
08-Sep-00	swab	5	bay 3 toilet area	wash basin tap handle	N
08-Sep-00	swab	5	bay 4 main bay	wash basin tap handle	N
08-Sep-00	swab	5	sluice-room	bed pan disposal unit	N
08-Sep-00	contact plate	6	shower-room	shower seat	N
08-Sep-00	contact plate	6	sluice-room	floor	N
08-Sep-00	contact plate	6	bay 4 toilet area	toilet seat	P
08-Sep-00	contact plate	6	bay 4 main bay	floor	N
08-Sep-00	contact plate	6	bay 3 toilet area	toilet seat	N
08-Sep-00	contact plate	6	bay 3 toilet area	floor	N
08-Sep-00	contact plate	6	bay 1 main bay	floor	N
08-Sep-00	contact plate	6	shower-room	floor	N
08-Sep-00	contact plate	6	bathroom	bath	N
08-Sep-00	contact plate	6	sluice-room	commode	P
08-Sep-00	contact plate	6	bay 2 main bay	floor	N
08-Sep-00	contact plate	6	bathroom	hoist sling	N
08-Sep-00	swab	6	sluice-room	bed pan disposal unit	N
08-Sep-00	swab	6	sluice-room	commode	N
08-Sep-00	swab	6	bathroom	toilet handle	N
08-Sep-00	swab	6	shower-room	shower seat	N
08-Sep-00	swab	6	shower-room	shower seat	N
08-Sep-00	swab	6	bay 1 main bay	locker	N
08-Sep-00	swab	6	bay 1 toilet area	toilet handle	N
08-Sep-00	swab	6	bay 2 main bay	locker	N

Date of sampling	Sampling method	Ward	Area sampled				Result
08-Sep-00	swab	6	bay	2	main bay	wash basin tap handle	N
08-Sep-00	swab	6	bay	3	toilet area	toilet seat	N
08-Sep-00	swab	6	bay	3	toilet area	toilet door handle	N
08-Sep-00	swab	6	bay	4	toilet area	toilet seat	N
08-Sep-00	swab	6	bay	4	toilet area	toilet door handle	N
08-Sep-00	swab	6	bathroom			ambulift	N
13-Oct-00	contact plate	5	bathroom			bed pan disposal unit	N
13-Oct-00	contact plate	5	shower-room			shower seat	N
13-Oct-00	contact plate	5	sluice-room			floor	P
13-Oct-00	contact plate	5	sluice-room			commode	N
13-Oct-00	contact plate	5	bathroom			floor	N
13-Oct-00	contact plate	5	bay	1	main bay	floor	N
13-Oct-00	contact plate	5	bay	1	toilet area	floor	N
13-Oct-00	contact plate	5	bay	2	main bay	floor	N
13-Oct-00	contact plate	5	bay	2	toilet area	toilet seat	N
13-Oct-00	contact plate	5	bay	3	main bay	locker	N
13-Oct-00	contact plate	5	bay	4	toilet area	shelf	N
13-Oct-00	contact plate	5	bay	4	main bay	floor	N
13-Oct-00	contact plate	5	SR	8	main room	armchair	N
13-Oct-00	contact plate	5	bay	3	main bay	floor	N
13-Oct-00	swab	5	SR	8	toilet area	toilet door handle	N
13-Oct-00	swab	5	bay	2	main bay	wash basin tap handle	N
13-Oct-00	swab	5	bay	2	toilet area	wash basin tap handle	N
13-Oct-00	swab	5	bay	3	main bay	patient trolley	N

Date of sampling	Sampling method	Ward	Area sampled				Result
13-Oct-00	swab	5	bay	3	toilet area	toilet handle	N
13-Oct-00	swab	5	bay	4	main bay	armchair	N
13-Oct-00	swab	5	bay	4	main bay	wheel chair	N
13-Oct-00	swab	5	bay	1	toilet area	toilet seat	N
13-Oct-00	swab	5	bay	1	main bay	monkey pole	N
13-Oct-00	swab	5	shower-room			shower door handle	N
13-Oct-00	swab	5	sluice-room			bed pan disposal unit	N
13-Oct-00	swab	5	bathroom			tap handle	N
13-Oct-00	swab	5	sluice-room			commode	N
13-Oct-00	swab	5	bathroom			toilet handle	N
13-Oct-00	contact plate	6	sluice-room			floor	P
13-Oct-00	contact plate	6	shower-room			floor	N
13-Oct-00	contact plate	6	bathroom			shelf	P
13-Oct-00	contact plate	6	bathroom			ambulift	N
13-Oct-00	contact plate	6	sluice-room			commode	N
13-Oct-00	contact plate	6	bay	4	toilet area	floor	N
13-Oct-00	contact plate	6	bay	3	main bay	mattress	P
13-Oct-00	contact plate	6	bay	4	main bay	floor	N
13-Oct-00	contact plate	6	SR	10	main room	locker	P
13-Oct-00	contact plate	6	bay	1	main bay	floor	N
13-Oct-00	contact plate	6	bay	3	toilet area	toilet seat	N
13-Oct-00	contact plate	6	bay	1	main bay	locker	N
13-Oct-00	contact plate	6	bay	2	main bay	locker	N
13-Oct-00	contact plate	6	bay	2	main bay	armchair	N

Date of sampling	Sampling method	Ward	Area sampled		Result
13-Oct-00	swab	6	bathroom	shower curtain	N
13-Oct-00	swab	6	bathroom	seat	N
13-Oct-00	swab	6	sluice-room	commode	N
13-Oct-00	swab	6	sluice-room	bed pan disposal unit	N
13-Oct-00	swab	6	shower-room	shower head	N
13-Oct-00	swab	6	bay 4 toilet area	wash basin tap handle	N
13-Oct-00	swab	6	bay 1 main bay	zimmer frame	N
13-Oct-00	swab	6	bay 1 toilet area	toilet seat	N
13-Oct-00	swab	6	bay 2 main bay	wash basin tap handle	N
13-Oct-00	swab	6	bay 2 main bay	patient trolley	N
13-Oct-00	swab	6	bay 3 toilet area	wash basin tap handle	N
13-Oct-00	swab	6	bay 4 toilet area	toilet handle	N
13-Oct-00	swab	6	bay 3 toilet area	toilet door handle	N
23-Oct-00	contact plate	5	bathroom	long sling	N
23-Oct-00	contact plate	5	sluice-room	commode	N
23-Oct-00	contact plate	5	SR 8 toilet area	floor	N
23-Oct-00	contact plate	5	bay 4 main bay	counter pane	N
23-Oct-00	contact plate	5	bay 1 main bay	floor	N
23-Oct-00	contact plate	5	bay 3 main bay	curtain screen	N
23-Oct-00	contact plate	5	bay 4 main bay	patient trolley	N
23-Oct-00	contact plate	5	sluice-room	cupboard	N
23-Oct-00	contact plate	5	bay 2 toilet area	toilet seat	N
23-Oct-00	contact plate	5	bay 2 main bay	floor	N
23-Oct-00	contact plate	5	bay 1 main bay	locker	N

Date of sampling	Sampling method	Ward	Area sampled		Result
23-Oct-00	contact plate	5	shower-room	shower seat	N
23-Oct-00	contact plate	5	bay 3 main bay	floor	N
23-Oct-00	contact plate	5	bathroom	shower screen	N
23-Oct-00	swab	5	SR 8 toilet area	toilet door handle	N
23-Oct-00	swab	5	bay 4 toilet area	wash basin tap handle	N
23-Oct-00	swab	5	bay 4 toilet area	toilet handle	N
23-Oct-00	swab	5	bay 3 toilet area	wash area	N
23-Oct-00	swab	5	bay 2 toilet area	toilet handle	N
23-Oct-00	swab	5	bay 2 toilet area	toilet door handle	N
23-Oct-00	swab	5	bay 3 main bay	seat	N
23-Oct-00	swab	5	bay 1 main bay	wash basin tap handle	N
23-Oct-00	swab	5	bay 1 main bay	door handle	N
23-Oct-00	swab	5	bathroom	ambulift	N
23-Oct-00	swab	5	shower-room	shower head	N
23-Oct-00	swab	5	bathroom	toilet handle	N
23-Oct-00	swab	5	sluice-room	bed pan disposal unit	N
23-Oct-00	swab	5	sluice-room	wash basin tap handle	N
23-Oct-00	contact plate	6	sluice-room	shelf	N
23-Oct-00	contact plate	6	sluice-room	draining board	N
23-Oct-00	contact plate	6	bathroom	shower screen	N
23-Oct-00	contact plate	6	SR 9 toilet area	floor	N
23-Oct-00	contact plate	6	bathroom	argio sling	P
23-Oct-00	contact plate	6	bay 4 main bay	paper towel dispenser	N
23-Oct-00	contact plate	6	bay 4 main bay	wash bowl	N

Date of sampling	Sampling method	Ward	Area sampled				Result
23-Oct-00	contact plate	6	bay	3	toilet area	cistern	N
23-Oct-00	contact plate	6	bay	3	main bay	floor	N
23-Oct-00	contact plate	6	bay	2	main bay	seat	N
23-Oct-00	contact plate	6	bay	2	main bay	locker	N
23-Oct-00	contact plate	6	bay	1	toilet area	floor	P
23-Oct-00	contact plate	6	bay	1	main bay	floor	N
23-Oct-00	contact plate	6	shower-room			shower seat	P
23-Oct-00	swab	6	sluice-room			bed pan disposal unit	N
23-Oct-00	swab	6	sluice-room			bed pan disposal unit	N
23-Oct-00	swab	6	bathroom			toilet handle	N
23-Oct-00	swab	6	bay	4	toilet area	wash basin tap handle	N
23-Oct-00	swab	6	bathroom			wash basin tap handle	N
23-Oct-00	swab	6	shower-room			shower door handle	N
23-Oct-00	swab	6	bay	1	toilet area	toilet seat	N
23-Oct-00	swab	6	bay	1	toilet area	toilet door handle	N
23-Oct-00	swab	6	bay	2	main bay	bed	N
23-Oct-00	swab	6	bay	2	main bay	bed	N
23-Oct-00	swab	6	bay	3	main bay	wash basin tap handle	N
23-Oct-00	swab	6	bay	4	main bay	seat	N
23-Oct-00	swab	6	SR	9	toilet area	toilet door handle	N
23-Oct-00	swab	6	bay	3	toilet area	wash basin tap handle	N
30-Nov-00	contact plate	5	bay	2	toilet area	toilet seat	N
30-Nov-00	contact plate	5	SR	9	toilet area	floor	N
30-Nov-00	contact plate	5	SR	9	main room	floor	P

Date of sampling	Sampling method	Ward	Area sampled			Result
30-Nov-00	contact plate	5	bay 4	toilet area	wall tiles	N
30-Nov-00	contact plate	5	bay 3	toilet area	seat	N
30-Nov-00	contact plate	5	bay 3	main bay	locker	N
30-Nov-00	contact plate	5	bay 2	main bay	floor	N
30-Nov-00	contact plate	5	bay 1	toilet area	floor	N
30-Nov-00	contact plate	5	sluice-room		shelf	N
30-Nov-00	contact plate	5	sluice-room		commode	N
30-Nov-00	contact plate	5	bathroom		ambulift	N
30-Nov-00	contact plate	5	bathroom		ambulift	N
30-Nov-00	contact plate	5	shower-room		shower seat	N
30-Nov-00	contact plate	5	bay 1	main bay	floor	N
30-Nov-00	contact plate	5	bay 1	main bay	floor	P
30-Nov-00	swab	5	bay 1	toilet area	toilet seat	N
30-Nov-00	swab	5	bathroom		equipment tray	N
30-Nov-00	swab	5	bathroom		ambulift	N
30-Nov-00	swab	5	shower-room		shower seat	N
30-Nov-00	swab	5	sluice-room		bed pan disposal unit	N
30-Nov-00	swab	5	sluice-room		commode	N
30-Nov-00	swab	5	bay 1	toilet area	toilet handle	N
30-Nov-00	swab	5	bay 2	toilet area	toilet door handle	N
30-Nov-00	swab	5	bay 2	main bay	wash basin tap handle	N
30-Nov-00	swab	5	bay 3	main bay	patient tray	N
30-Nov-00	swab	5	bay 3	main bay	bed	N
30-Nov-00	swab	5	bay 4	toilet area	floor	N

Date of sampling	Sampling method	Ward	Area sampled		Result
30-Nov-00	swab	5	SR 9 main room	wash basin tap handle	N
30-Nov-00	swab	5	SR 9 main room	locker	N
30-Nov-00	contact plate	6	bathroom	bath	N
30-Nov-00	contact plate	6	bay 1 toilet area	floor	N
30-Nov-00	contact plate	6	bay 1 main bay	locker	N
30-Nov-00	contact plate	6	shower-room	shower floor	N
30-Nov-00	contact plate	6	bathroom	toilet seat	N
30-Nov-00	contact plate	6	sluice-room	commode	N
30-Nov-00	contact plate	6	sluice-room	floor	N
30-Nov-00	contact plate	6	SR 6 toilet area	commode	N
30-Nov-00	contact plate	6	bay 2 toilet area	floor	N
30-Nov-00	contact plate	6	SR 6 main room	floor	N
30-Nov-00	contact plate	6	SR 6 main room	locker	N
30-Nov-00	contact plate	6	bay 4 toilet area	floor	N
30-Nov-00	contact plate	6	bay 4 main bay	floor	N
30-Nov-00	contact plate	6	bay 3 toilet area	floor	N
30-Nov-00	contact plate	6	bay 3 main bay	floor	N
30-Nov-00	swab	6	bay 1 main bay	bed	N
30-Nov-00	swab	6	bay 3 toilet area	toilet seat	N
30-Nov-00	swab	6	sluice-room	bed pan disposal unit	N
30-Nov-00	swab	6	sluice-room	commode	N
30-Nov-00	swab	6	bathroom	wash basin tap handle	N
30-Nov-00	swab	6	bathroom	toilet handle	N
30-Nov-00	swab	6	bay 2 toilet area	toilet handle	N

Date of sampling	Sampling method	Ward	Area sampled			Result
30-Nov-00	swab	6	bay 1	toilet area	toilet door handle	N
30-Nov-00	swab	6	bay 3	main bay	wash basin tap handle	N
30-Nov-00	swab	6	bay 4	toilet area	toilet handle	N
30-Nov-00	swab	6	bay 4	toilet area	toilet door handle	N
30-Nov-00	swab	6	SR 6	toilet area	commode	N
30-Nov-00	swab	6	SR 6	main room	bed	N
30-Nov-00	swab	6	shower-room		shower seat	N

APPENDIX 4
The number of patients exposed to each of the possible risk factors investigated by logistic regression modelling.

Risk Factor	*C. difficile* status		
	Cdc- n=272	Cdc+ n=66	Cdt+ n=34
Gender	90	29	11
Neoplasia	29	6	1
Colonic disease	33	6	1
Leukaemia	2	2	0
Laxative use	134	25	11
NG/PEG use	2	2	2
Steroid use	18	3	4
Antacid use	65	19	13
Community admission	154	26	13
Hospital admission	100	40	20
Nursing home admission	18	0	1
Any antibiotic use	112	37	27
Amoxycillin	10	2	7
Co-amoxiclav (augmentin)	67	20	1
Other penicillins	12	5	16
Ceftriaxone	2	5	4
"Other" cephalosporins	3	2	6
Macrolide	15	10	5
Quinolones	26	7	6
Other parenteral (IV/IM) antibiotics	25	4	3
Trimethoprim	12	3	6
Cephalosporins	5	7	6

Cdc- = *C. difficile* culture and toxin negative
Cdc+ = *C. difficile* culture positive (toxin negative)
Cdt+ = *C. difficile* culture and toxin positive.

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Variation in the surface layer proteins of *Clostridium difficile*

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Abstract

Surface layers (S-layers) form regular crystalline structures on the outermost surface of many bacteria. *Clostridium difficile* possesses such an S-layer consisting of two protein subunits. Treatment of whole cells of *C. difficile* with 5 M guanidine hydrochloride revealed two major proteins of different molecular masses characteristic of the S-layer on SDS–PAGE. In this study 25 isolates were investigated. A high degree of variability in the molecular mass of the two S-layer proteins was evident. Molecular masses ranged from 48 to 56 kDa for the heavier protein and from 37 to 45 kDa for the lighter protein. A further protein component of 70 kDa was detectable in all isolates. No cross-reaction was seen between the two major proteins from isolates that produced different S-layer patterns, and most S-layer proteins from isolates with the same or similar banding patterns did not cross-react. The S-layer proteins, when detected by a combination of Coomassie blue staining and immunoblotting, are a useful marker for phenotyping.

Keywords: Surface layer protein; Surface layer; Phenotyping; Guanidine hydrochloride; *Clostridium difficile*

1. Introduction

Clostridium difficile is the most common aetiological agent of antibiotic-associated diarrhoea and pseudomembranous colitis. There are a number of virulence factors associated with the organism including two main toxins that are responsible for the major pathology of the disease [1].

C. difficile expresses two major cell surface proteins, known as S-layer proteins. This crystalline cell surface layer was first described by Kawata et al. in 1984 [2], who reported that it was composed of two proteins with different molecular masses. Sharp and Poxton [3] later showed that these proteins varied between strains both in molecular mass and immunogenic reactivity. S-Layers have been reported on many bacteria including numerous pathogens and it is assumed that they could have a role in virulence [4]. These protein components cover the entire cell surface as a regular array, and are therefore ideal candidates for bacteria–host interactions. S-Layer proteins have been described as virulence factors for several human pathogens, including *Wolinella recta* [5] and *Bacillus cereus* [6]. The S-layer of *C. difficile* may contribute to the virulence of the organism by promoting adherence and colonisation, or by evasion of the immune system.

The aims of this study were to investigate the variability of the S-layer proteins between different strains of *C. difficile*, to determine the relative immunoreactivity of the S-layer proteins and to assess the expression of S-layer proteins as a useful phenotypic marker for epidemiological studies.

Preliminary work on which this paper is based was presented at the meeting of the Anaerobe Society of the Americas in Buenos Aires 1998 [7].

2. Materials and methods

2.1. Bacterial isolates

Twenty-five isolates of *C. difficile* were examined. Each of the isolates had been designated a 'Delmée' serotype or a 'Cardiff' ribotype and were kindly donated by Dr Jon Brazier, Anaerobe Reference Unit, Cardiff. A reference strain, NCTC11223 (mprl 2520), was also included. These are shown in Table 1.

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Table 1
The 25 strains used in the study, showing their laboratory number and the serotype or ribotype for each strain

Strain name	Serotype	Ribotype
mprl 4196	A	
mprl 4197	B	
mprl 4198	C	
mprl 4199	D	
mprl 4200	F	
mprl 4201	G	
mprl 4202	H	
mprl 4203	I	
mprl 4204	K	
mprl 4205	X	
mprl 4206	A2	
mprl 4207	A3	
mprl 4208	A4	
mprl 4209	A5	
mprl 4210	A6	
mprl 4211	A7	
mprl 4212	A8	
mprl 4213	A9	
mprl 4214	A10	
mprl 4215		10
mprl 4216		23
mprl 4218		106
mprl 4219		56
NCTC 11223	n.d.	
MPRL 1128	n.d.	

n.d., not determined

2.2. Extraction of S-layer proteins from whole bacterial cells

Overnight cultures of *C. difficile* were grown in protease–peptone yeast extract medium [8], pH 7.1, supplemented with sodium carbonate 0.04% w/v and cysteine hydrochloride 0.075% w/v [9]. The cultures were incubated anaerobically with 10% CO_2 overnight at 37°C.

Bacteria were harvested at $6000 \times g$ for 15 min and washed twice in 4 ml of phosphate buffered saline. The extracted cells were resuspended in 0.3 ml of 5 M guanidine hydrochloride and shaken for 2 h at room temperature. The extracted bacteria were removed by centrifugation twice for 2 min at $16\,000 \times g$ and discarded. The guanidine hydrochloride was removed from the protein extract by dialysis against 6.25 mM Tris–HCl buffer, pH 6.8 using a Spectra/Por® Microdialyzer with a membrane of 10 000 MWCO and the dialysed extract was stored at −20°C.

2.3. SDS–PAGE and immunoblotting

The protein extract (1 mg ml^{-1}) was mixed with an equal amount of double strength SDS–PAGE buffer [10] and heated in a 100°C boiling bath for 3 min. Extracts were analysed on 10% separating gels, with 4% stacking gels using the buffer system of Laemmli [10]. An initial voltage of 60 V was used to pull the proteins through the stacking gel. The voltage was increased to 150 V and run for approx. 4 h until the dye front had moved through the separating gel. The separated proteins were stained with Coomassie blue [11] or transferred to a nitrocellulose membrane for immunoblotting.

Briefly, the Tris–glycine transfer method of Towbin et al. [12] as described in detail by Hancock and Poxton [11] was followed. The separated guanidine hydrochloride extracts were transferred to a nitrocellulose membrane (0.2 μm pore size, Schleicher&Schuell) in Tris, glycine, methanol buffer, pH 8.3 at 40 mA for 18 h at 4°C.

After transfer and washing the nitrocellulose was blocked with 3% (w/v) gelatin in Tris buffered saline (TBS, pH 7.5) for 45 min. The nitrocellulose membrane was incubated with *C. difficile* rabbit antiserum, raised to whole UV-killed cells (see Section 2.4), diluted 1/200 in gelatin 1% (w/v) in TBS. This was incubated at room temperature for 3 h. After washing twice for 10 min in Tween TBS (TTBS) the membrane was incubated in anti-rabbit IgG–horseradish peroxidase (HRP) diluted 1/2000 at room temperature for 1 h. After two further washes in TTBS, the nitrocellulose membrane was washed three times in distilled water. The nitrocellulose was placed into HRP developer and the colour developed within 5–30 min. The development was stopped by several washes in distilled water.

2.4. Rabbit antisera

Antisera were raised in rabbits to five different strains of *C. difficile* (mprl strains 683, 1123, 1128, 604 and NCTC 11223) using whole UV-killed *C. difficile* cells as described previously for *Bacteroides* spp. [13,14].

2.5. Calculation of molecular masses

Molecular masses of the S-layer proteins were calculated by computerised analysis of protein patterns using Phoretix™ gel analysis 1-D software. Novex, Mark12™ molecular mass standards were used as calibrations for the calculation of molecular masses.

3. Results

3.1. SDS–PAGE profile of guanidine hydrochloride protein extracts

Whole *C. difficile* cells treated with 5 M guanidine hydrochloride were shown to produce two major and one minor protein bands on SDS–PAGE. Fig. 1 shows the patterns produced by 15 different isolates. The two major protein bands are characteristic of the S-layer proteins described by Kawata et al. [2]. There is a high degree of variation in the S-layer protein profiles between the isolates. The larger of the two proteins varies in molecular

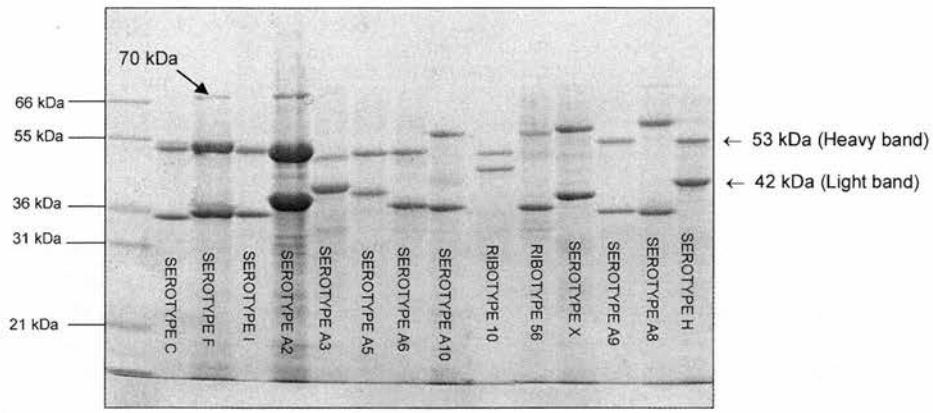

Fig. 1. SDS–PAGE of S-layer proteins of *C. difficile* isolates. Lane 1: molecular mass markers. Other lanes are as indicated on the gel.

mass from 48 to 56 kDa and the smaller from 37 to 45 kDa. A third, minor band is visible in some of the extracts on the Coomassie blue stained gel. It has a molecular mass of 70 kDa and appears to be conserved throughout these isolates.

3.2. Calculation of the molecular masses of the S-layer proteins

Each of the isolates used in this study was designated a four-digit 'type number' based on the molecular masses of the two S-layer proteins; e.g. 5336, where 53 is the molecular mass of the heavier band and 36 of the lighter band, in kDa.

The isolates were grouped on the basis of the 'type number', and this is shown in Table 2. A total of 12 groups were assigned to the 24 isolates investigated.

3.3. The antigenic nature and cross-reactivity of the C. difficile S-layer proteins

Multiple immunoblots using each of the five antisera showed very little cross-reaction between the S-layer proteins of the different strains even when the protein profiles were identical to the pattern produced by the strain to which the antiserum was raised. The protein of molecular mass 70 kDa, corresponding to the band of that mass on the Coomassie stain, appears in all isolates and cross-reacted with all antisera tested. It appears to be an antigen common to all strains. All of the results are not shown, but results of two of the antisera tested with representative isolates are shown in Fig. 2.

Seven isolates (serotypes C, G, K, I, F, A9 and NCTC11223) with very similar S-layer protein profiles belonged to type '5336'; the Coomassie blue stained gel of six of these is shown in Fig. 2A. Immunoblotting was performed on the S-layer proteins from these seven isolates employing two different antisera: one raised against NCTC11223 and the other raised against MPRL 1128, a strain that cross-reacts with serotype F. The antiserum raised against NCTC11223 cross-reacted with the S-layer proteins from isolates representing serotypes C and K only (Fig. 2B). The antiserum raised against the serotype F strain reacted only with the S-layer proteins from the isolate representative of serotype F (Fig. 2C).

The S-layer proteins appear to display distinct antigenic differences between isolates and the smaller of the S-layer proteins exhibits a stronger reaction in the immunoblots. Immunoblotting can further discriminate between isolates with S-layer proteins of similar molecular mass.

Table 2
The 23 sero/ribotyped isolates grouped on the basis of their 'S-type number'

S-Type number	Serotype	Ribotype
48 45	A	23
50 41	A3	
50 45	D	10
51 37	A6	
51 40	A5	
51 42	A4	
52 39	A2	
53 36	C, G, I, K, F, A9 and NCTC11223	
53 42	H	106
55 43	A7	
56 35	A8	
56 37	B, X, A10	56

4. Discussion

Numerous methods exist for the typing of *C. difficile*. Historically these have been phenotypic methods including SDS–PAGE, immunoblotting [9] and serotyping [15]. More recently genotypic methods such as ribotyping have been used for typing *C. difficile* [16]. However, genotypic methods do not provide any data on the presence or pathogenic functions of virulence factors. The typing method described in this paper, although phenotypic, offers important details of the cell surface structures which are circumstantially related to virulence in the same way as serotyping [17].

Treatment of whole washed *C. difficile* cells with guani-

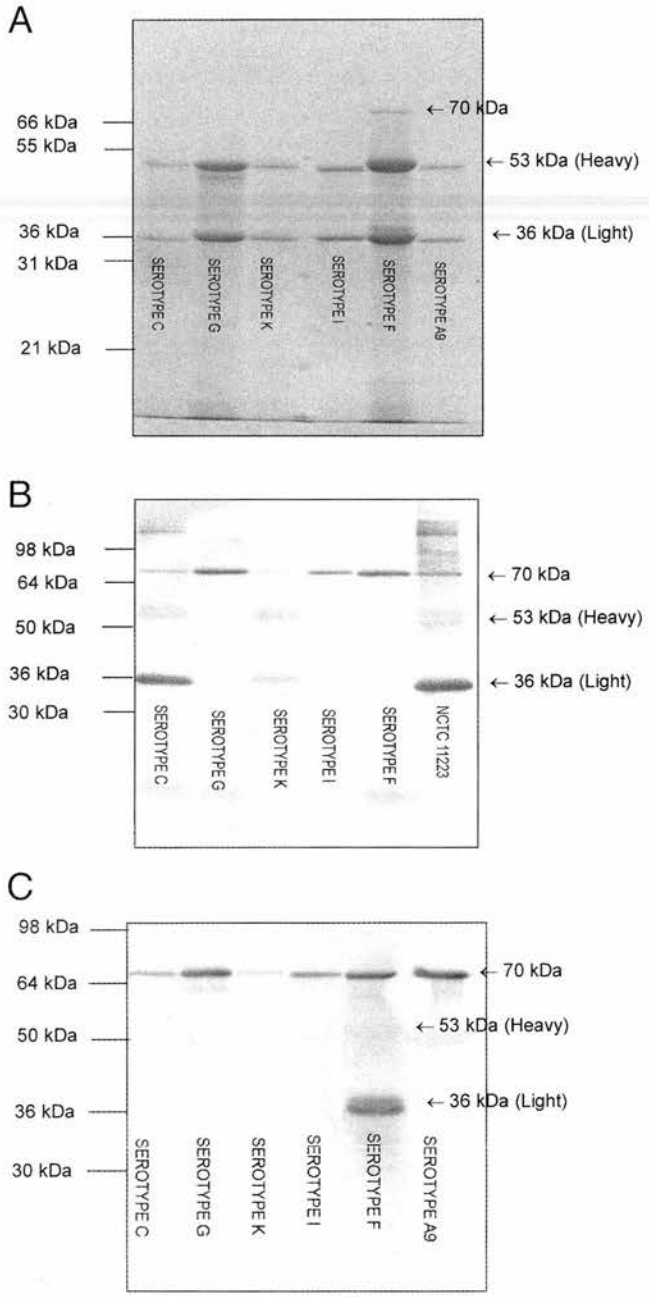

Fig. 2. PAGE and immunoblot analysis of S-layer proteins from a selection of isolates belonging to group '5336'. The lanes are as indicated on the gel. A: Coomassie blue stained SDS–PAGE. B: immunoblot using antiserum raised to NCTC11223. C: immunoblot using antiserum raised to MPRL 1128 (a serotype F strain).

dine hydrochloride extracts the two major cell surface proteins. A study by Kawata et al. [2] showed that treatment of the outer cell wall extracts with urea or guanidine hydrochloride produced almost identical protein extracts. More recently, Cerquetti et al. [18] demonstrated that complex preparation of cell walls was not required and treatment of whole cells with urea led to the extraction of S-layer proteins. We therefore conclude that these are the S-layer proteins.

There is a high degree of variation in the S-layer protein profiles of the different serotypes and ribotyped strains. Further analysis using immunoblotting can distinguish strains with similar protein profiles as there appears to be little antigenic cross-reactivity between strains unless they share identical S-layer proteins. This agrees with earlier work which stated that any single strain only reacts with homologous antiserum [13,19].

Immunoblotting suggests that the S-layer protein of lower molecular mass may be more immunogenic than the larger of the two proteins, although this interpretation must be taken with caution as immunoblotting is not quantitative. It is tempting to speculate that the S-layer proteins have a role in virulence, but this and the significance of the immune response to the S-layer proteins have yet to be determined.

Comparison of SDS–PAGE protein profiles is a technique that has been used extensively in epidemiological studies of *C. difficile*. Poxton et al. [9] used immunochemical fingerprinting of EDTA extracted proteins to investigate an outbreak of antibiotic-associated colitis and diarrhoea. The method described in the present paper yields only the proteins of interest. The resulting SDS–PAGE protein profiles are extremely simple and are easily interpreted by computer software analysis. The stability of the surface proteins of *C. difficile* isolates was investigated both in vitro and in vivo, and only minor variations were observed [13]. In addition, our unpublished data show a good correlation between this typing method and PCR ribotyping. The profiles are clear and reproducible and could complement other typing techniques in epidemiological studies.

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The pathogenicity of *Clostridium difficile*

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It is now well established that the major virulence factors of *C. difficile* are the two toxins A and B. However, the organism possesses an array of other putative virulence factors that may be important for localisation within the colon, and in evasion of the immune system. It has been observed that certain types of *C. difficile* are more commonly found causing disease than others, and this seems to be independent of toxin production. Is this simply a reflection of their abundance in the hospital environment, or is it due to their virulence determinants? This review covers our current knowledge of the modes of action of toxins A and B at the cellular and molecular level. Many unanswered questions are posed that require answers before we can fully understand the pathogenic mechanisms of the organism and be in a position to manage better the spectrum of diseases it causes.

Keywords Toxin A, toxin B, antibiotic-associated colitis, *Clostridium difficile*, pathogenicity

Clin Microbiol Infect 2001; 7: 421–427

INTRODUCTION

The spectrum of disease and its pathology

Clostridium difficile is a commonly isolated organism from fecal specimens obtained from neonates and the elderly. Often its carriage is asymptomatic, and this is especially true in the neonate. However, in the elderly, it is often associated with disease symptoms that range from mild self-limiting diarrhea to serious diarrhea, with or without pseudomembrane formation (pseudomembranous colitis; Figure 1), and with the possibility of life-threatening complications such as toxic megacolon, perforation and peritonitis.

Antibiotics and the normal gastrointestinal microbiota

The proposed sequence of events that precipitate *C. difficile* disease are as follows: on exposure of the gut to antibiotics, the microbiota becomes disrupted and colonisation resistance is compromised. The gut is then susceptible to colonisation by +*C. difficile*. The organism is acquired in most cases from an exogenous source – either from an infected individual, from a contaminated health care worker, or indirectly from a contaminated environment. Once ingested *C. difficile* evades immune responses, multiplies in the colon and produces toxins A and B. The characteristic pathology then results.

Virulence factors of *C. difficile*

C. difficile is typical of its genus: it is an anaerobic, Gram-positive, spore-forming bacillus that produces toxins. The two toxins A and B are commonly referred to as the enterotoxin (toxin A) and the cytotoxin (toxin B). This terminology originated from the observed actions of these toxins: demonstration of fluid accumulation in intestinal loop models and the cytopathic effects on tissue culture monolayers, respectively. These investigations were done before the action at the molecular level was well understood. However, as is described below, both toxins have a great deal in common.

Other toxins have been identified, in particular the bipartite, ADP-ribosylating toxin, which is described in more detail in an accompanying review [1].

The role of other virulence factors is much more speculative. Adhesins have been proposed as being important but their relevance in the colon, and their identity, is still not assured. Several extracellular enzymes are produced that do have effects in vitro, but their role in pathogenesis is not well defined. Presumably these enzymes do have a role in the normal physiological processes in the gastrointestinal (GI) tract and may be crucial for the normal survival of the organism, giving it an advantage when the normal GI microbiota have been disturbed following antibiotic usage.

C. difficile is somewhat unusual in that it has an outer cell coat termed the S-layer. This consists of two polypeptides that together form a regular, crystalline array over the whole surface

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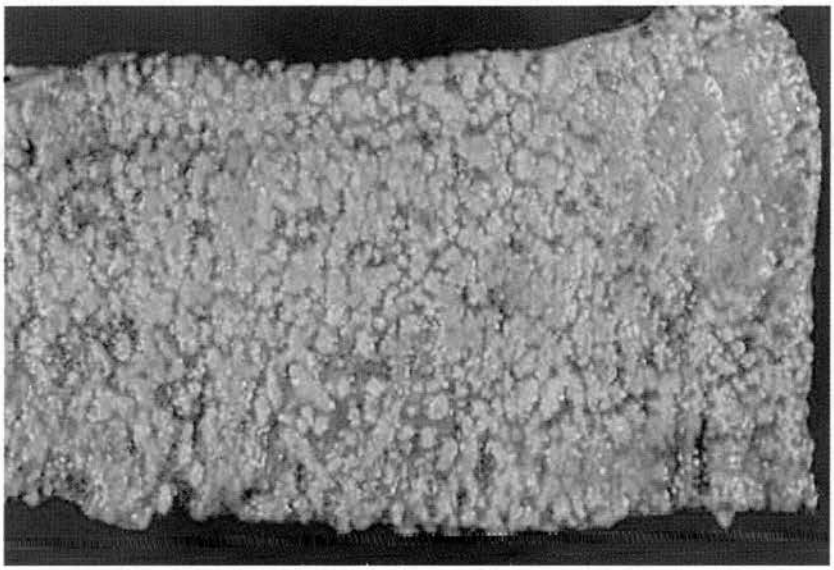

Figure 1 Pseudomembranous colitis, post mortem specimen.

of the bacterium. The S-layer is proposed to be a virulence factor and is discussed in more detail later.

THE TOXINS AND HOW THEY WORK

The majority of toxigenic strains produce both the A and B toxins. In summary, their mode of action is similar: they are endocytosed by the cell, they affect the actin cytoskeleton and they result in cell death. They also induce the production of tumor necrosis factor-alpha (TNFα) and proinflammatory interleukins (ILs) which contribute to the associated inflammatory response and pseudomembrane formation.

Toxin A causes necrosis, increased intestinal permeability and inhibition of protein synthesis. Toxin A also affects phospholipase A2, thereby producing prostaglandins and leukotrienes. Toxin A damages villous tips and brush border membranes and complete erosion of the mucosa may result. A viscous, bloody fluid is produced in response to this tissue damage. However, in the case of toxin B, there is no noticeable enterotoxic activity but it is lethal to cells in vitro. Therefore Toxin B is thought to become effective once the gut wall has been damaged.

The structure of toxins A and B

The two major toxins are coded on a pathogenicity locus (Figure 2). The products of transcription and translation are extremely large single-chain peptides with molecular masses of 308 kDa for toxin A and 270 kDa for toxin B [3]. There are three functional domains to these toxins (Figure 3). The toxins

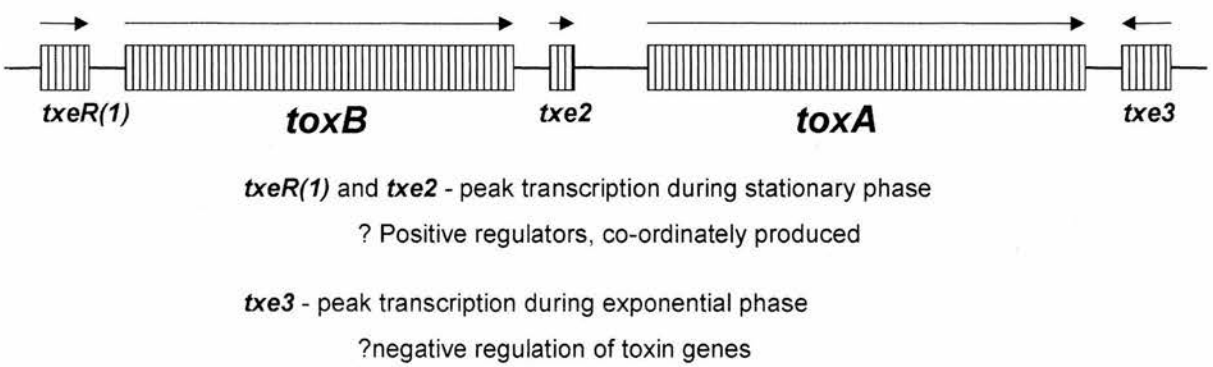

Figure 2 The pathogenicity locus of *Clostridium difficile* (modified from ref. 2).

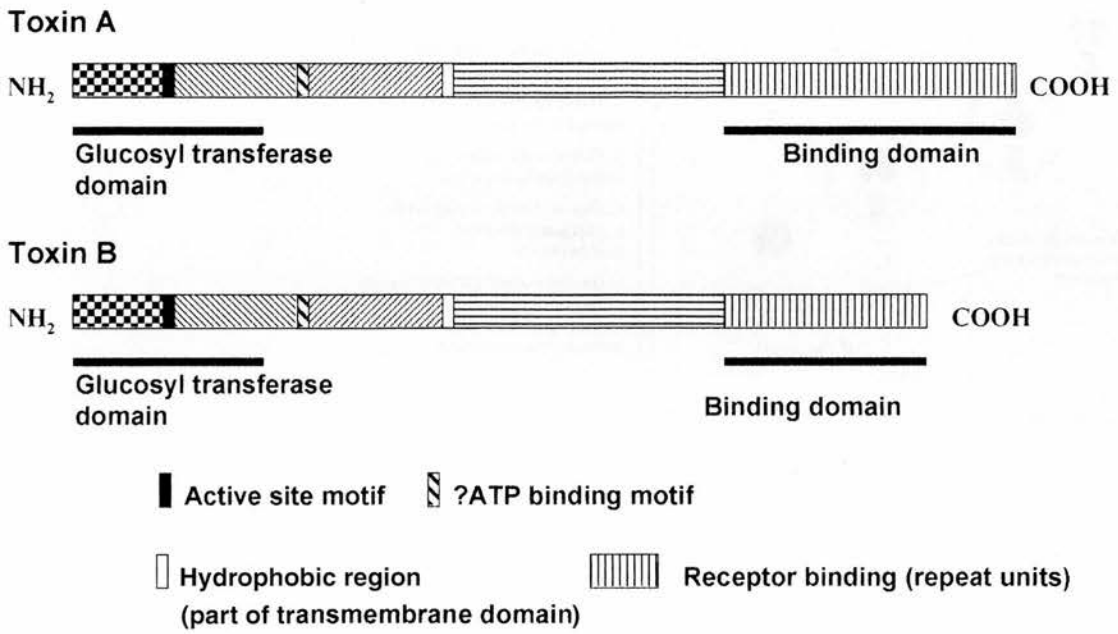

Figure 3 The structure of *Clostridium difficile* toxins A and B (modified from ref. 2).

are 50% identical at the amino acid level and have similar primary structures. Hoffman et al. [4] noted that the enzyme and cytotoxic activity of toxin B was to be found at the toxin's N-terminus, which also holds the enzyme and cytotoxic activity of toxin A. The middle section of both toxins includes a transmembrane domain, which is thought to encode for the translocation of the toxin into the cytosol, but this has yet to be proven [5]. The C-terminal of the toxin encompasses the receptor-binding domain and is constructed of repetitive peptide elements.

Action of toxins A and B

The carboxy terminal of toxin A forms binding domains for carbohydrate structures that occur on the surface of the epithelium. Toxin B binds to cells that are not covered by a thick carbohydrate matrix. They then enter the cell by endocytosis [6]. Both toxins require passage through an acidic intracellular compartment in order to intoxicate cells. This route is not known for toxin A, but toxin B is believed to be delivered by lysosomes and is then released into the cytosol.

The major effect of toxins A and B is the disruption of the actin cytoskeleton. Cells intoxicated by these proteins show a retraction of cell processes and a rounding of the cell body. This is due to the disassembly of filamentous F-actin and an increase in G-actin prior to cell rounding [7]. Very few toxin molecules are required to produce cell rounding. It has been proposed that *C. difficile* toxins act enzymatically within cells, modifying proteins that regulate actin polymerisation and fiber assembly. These proteins are known as the Rho proteins, a subfamily of the Ras-family of GTPases [7]. The mechanisms of action of either toxin are summarised in Figure 4.

In the diseased state, the colonic epithelium is the major target of *C. difficile* toxins. They cause disruption of the barrier function by opening the tight junctions. This effect is not merely caused by the breakdown of actin filaments but by the inactivation of the Rho function to regulate tight junction complexes. These barrier-disrupting effects of toxin A and B increase the colonic permeability, the basis of watery diarrhea, which is a typical feature of *C. difficile* antibiotic-associated diarrhea (Figure 5).

Apoptosis of enterocytes

A study by Fiorentini et al. [8] provided the first experimental evidence that cultured intestinal cells exposed to toxin B showed all the features of apoptosis. A study by Mahida et al. [9] showed the same effect being caused by toxin A. All cells undergo apoptosis at some point, as this controlled cell death is an important feature of tissue development and homeostasis, keeping the number of functional cells in balance in the body. Apoptosis can be identified as being different from cell necrosis by distinct morphological alterations. These alterations come in the form of nuclear condensation and fragmentation, cell shrinkage and the absence of inflammation [8]. In monolayers of cell cultures, apoptosis can be induced by inhibition of cell adhesion and of anchorage-dependent cell spreading. Toxin B is capable of both inhibition of anchorage and cell spreading.

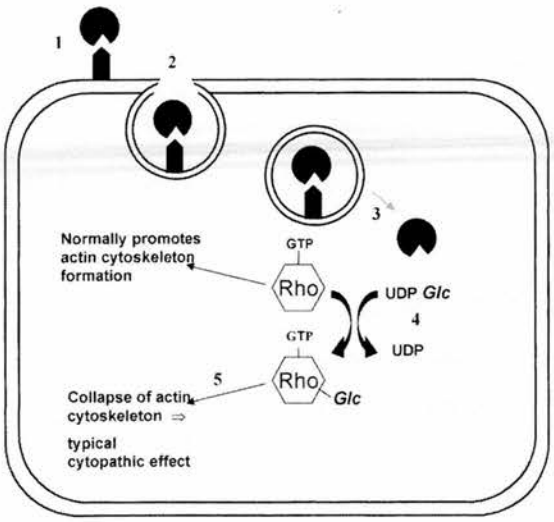

Figure 4 Simplified scheme for the action of *Clostridium difficile* toxins on cells (modified from ref. 6).

However, it was found that apoptosis was not only caused by toxin B inhibiting cell adhesion due to actin depolymerisation but that Rho proteins themselves may play an important role in the regulation of apoptosis under normal conditions [8]. Toxin B, therefore, can be seen as an inducer and not as the cause of apoptosis. It is the effects of toxin B on the Rho proteins that cause the abnormal activation of the apoptotic system. With toxin A, it is thought that apoptosis occurs because the epithelial cells are denied anchorage to the basement membrane.

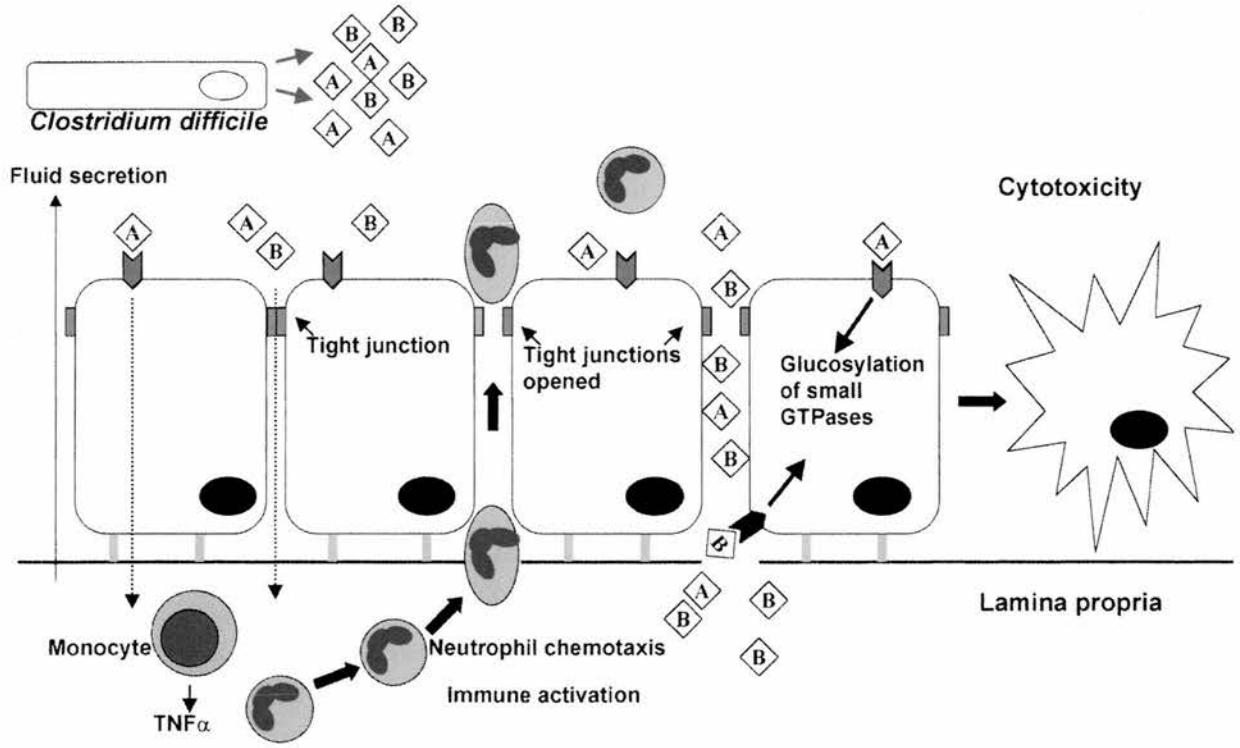

Figure 5 Actions of *Clostridium difficile* toxins A and B on intestinal epithelium (modified from ref. 6).

Differences in cytotoxic potencies in toxin A and B

To cause pseudomembranous colitis, both toxins A and B are normally required. The two toxins intoxicate cultured cell lines by the same mechanism but when it comes to potency, toxin B is around a 1000 times more potent than toxin A. Toxin B has at least a 100-fold higher enzymatic activity than toxin A, and this is believed to be the main determinant in the difference in cytotoxic potency.

Due to the low enzymatic potency of toxin A, it has been proposed that glucosylation of the Rho-proteins may not be the primary in vivo effect of this toxin. Some believe neuronal involvement may be a possible answer to enterotoxic effects of toxin A [10]. The suggestion is that the pathophysiological process is triggered by a transepithelial signal to neuroimmune cells that is triggered by the binding of toxin A to the intestinal mucosa. The modification of Rho proteins by toxin A and B would then play a secondary, but important, role in exacerbating mucosal inflammation and destruction. This theory agrees with an experiment done by Riegler et al. [11], where toxin B was shown to be more potent than toxin A in damaging human colonic epithelium in vitro. The mucosal strips were devoid of enteric nerves and thus toxin B was shown to be 10 times more effective in causing damage.

ACTIVATION OF THE IMMUNE SYSTEM

Colitis is characterised by a massive influx of neutrophils into the colonic mucosa, and in pseudomembranous colitis there is an acute inflammatory infiltrate with microabscesses and pseudomembranes rich in neutrophils [12]. The movement of neutrophils from circulating blood to the site of injury is a crucial event during the inflammatory process. IL-1, IL-8, TNF and leukotriene B4 are products of resident cells and are thought to be involved in neutrophil infiltration into the inflamed site.

Both toxins stimulate the release of TNFα from cultured monocytes. Toxin B was again found to be 1000 times more potent than toxin A in this system [12]. Both toxins also activate monocytes and macrophages in the lamina propria in vitro to release IL-8. This causes neutrophil extravasation and tissue infiltration by creating a chemotactic gradient that induces neutrophil migration to the site of mucosal inflammation [13]. Using mast cell-deficient mice, Pothoulakis et al. [14] demonstrated the importance of mast cells for neutrophil recruitment and fluid secretion induced by toxin A in vivo. Isolated mast cells were also shown to respond to toxin A by releasing TNFα. This activation could be inhibited with a specific antagonist to substance P. Substance P is a peptide found in gut tissue and in the CNS that acts as a neurotransmitter. This suggests that toxin A activates mast cells via the release of substance P from adjacent sensory neurons [15]. This can also be seen as another point marking toxin A activity with neuronal stimuli.

Neutrophil recruitment appears to be an essential step in the pathogenesis of *C. difficile* toxin-induced intestinal injury as biopsy specimens from patients with *C. difficile* colitis show marked vascular congestion, neutrophil infiltration of the lamina propria and inflammation. Although it was shown by Calderon et al. [15] that toxin A was able to activate neutrophils, mast cells and macrophages in vitro, there is still some speculation as to how this works in vivo due to the large size of the toxin. Toxin A can cause detachment and apoptosis of enterocytes and so, in this disrupted epithelium, toxin A may diffuse and interact with the inflammatory cells in the lamina propria. Also, localised areas of injury and inflammation may result from cell rounding that would cause breaches in the colonic epithelium through which tiny amounts of toxin A and B can pass. These small amounts cannot directly activate neutrophils but may activate tissue macrophages to produce IL-8 and other proinflammatory cytokines. Once the inflammatory cascade is initiated, it can result in a marked acute inflammatory cell infiltration, further mucosal injury and focal pseudomembrane formation [13].

IMMUNITY AND HOST DEFENCES

Innate mechanisms

Probably the best defence against infection by *C. difficile* is an intact normal bowel microbiota – preventing establishment of *C. difficile* by colonisation resistance [16]. Normal gut motility and an effective gastric acid barrier are no doubt also important [17].

Acquired immune mechanisms

Experiments with hamsters suggest that systemic IgG to toxin A – induced by vaccination – is protective [18], and secretory (IgA) antibodies to toxins A and B may be protective as demonstrated by protecting hamsters fed milk from immunised mothers. However, this is an area still requiring a great deal of work, especially to determine the potential for immunisation in humans [17].

PHENOTYPIC VARIATION

There is a degree of phenotypic variation between strains of *C. difficile*. In respect to pathogenicity, it seems that non-toxigenic strains can be considered non-pathogenic or avirulent. However, it is well accepted that there are degrees of virulence between strains. The discovery of virulent strains of toxin A-negative/toxin B-positive phenotype indicated that toxin A was not essential for virulence. How these strains cause

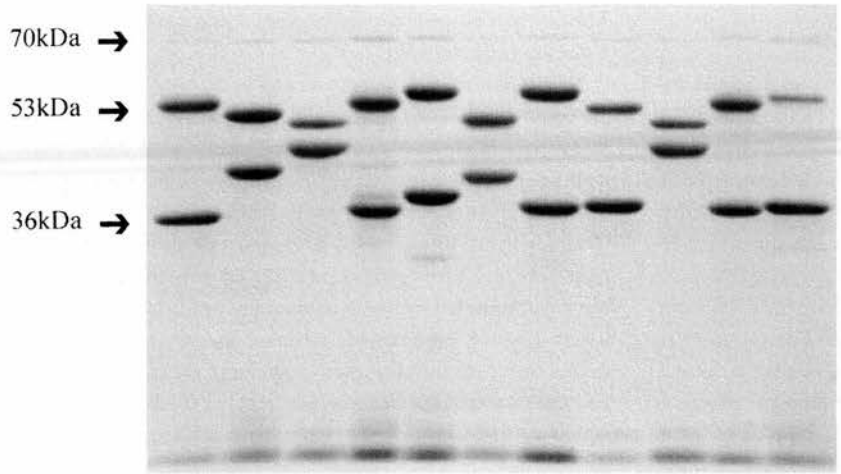

Figure 6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of S-layer proteins from 11 different strains of *Clostridium difficile*. The S-layer proteins were extracted from whole bacteria with guanidine hydrochloride.

disease is not readily apparent but based on the discussion above there is so much similarity between toxins A and B that once there is any damage to the mucosal cell layer then either could cause symptoms.

In common with several pathogens, *C. difficile* has an S-layer covering its entire surface. We have proposed recently that this might have a role in virulence [19]. There is certainly a degree of correlation between serotype and virulence that is independent of toxin production: certain serotypes are more often associated with disease. Serotype correlates well with S-type and also to ribotype [19 and unpublished data]. Figure 6 shows an example of the S-layer proteins extracted from different strains with guanidine hydrochloride, and each strain belongs to a different serotype.

UNANSWERED QUESTIONS

Despite knowing a great deal about the mode of action of the major toxins, and understanding the epidemiology of the pathogen, many questions remain unanswered. For some, there are partial answers but, in the opinion of the authors, none of the following are yet answered fully:

Why are infants not affected?

Do the toxins have any role in the healthy intestine?

Is immunity to cell surface components protective – would whole cell vaccines be a possibility?

Why are some types much more virulent than others?

What is the molecular basis of serotype?

Why are there so many S-types?

What is the genetic basis for S-layer peptide variation?

It is generally agreed that *C. difficile*-associated disease is increasing worldwide, and our last question, 'Have super strains evolved?', remains unanswered. Is the increase purely because the organism – as spores – becomes persistent in the environment of the elderly patient, where it is maintained by constantly being passaged in susceptible individuals, or have previously harmless strains from neonates acquired virulence attributes, and are more persistent, more virulent strains evolving?

ACKNOWLEDGMENTS

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Typing of *Clostridium difficile*

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Clostridium difficile is primarily recognised as a nosocomially acquired pathogen manifesting in gastrointestinal disease subsequent to the patient receiving broad-spectrum antibiotics. Infection can be sporadic, but outbreaks commonly occur within a ward or hospital as a result of cross-infection. Since the 1980s, the epidemiology of *C. difficile* disease has been studied by the application of many different typing or fingerprinting methods; these, and the lessons learned, are reviewed herein.

Keywords *Clostridium difficile*, typing

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PHENOTYPIC TYPING METHODS

Early methods of typing *Clostridium difficile* were, of necessity, based on phenotypic properties such as antibiograms. In one of the first documented outbreak investigations, Burdon et al. [1] found a common resistance pattern to three antibiotics in isolates from cases on a surgical ward that were distinct from isolates in the rest of the hospital. However, this approach is at best only rudimentary, and a more detailed approach was tried by Wüst et al. [2] who combined plasmid analysis, soluble protein polyacrylamide gel electrophoresis (PAGE), immunoelectrophoresis of extracellular antigens and antibiograms to 16 isolates from related cases of *C. difficile* infection. Using these methods they showed that 12 of the 16 strains were indistinguishable, providing strong evidence that cross-infection had taken place. Sell et al. [3] used a combination of bacteriocin and bacteriophage typing methods, with a high percentage of strains being non-typeable. Immuno-chemical fingerprinting of EDTA-treated cell extracts of *C. difficile* was evaluated [4], and Nakamura et al. [5] were the first investigators to use serum agglutination as a typing method by raising three antisera against *C. difficile*. This method could differentiate four distinct serovars among 79 isolates from healthy carriers. Delmée's group [6] improved this method and developed a serotyping scheme that could recognise 19 distinct sero-groups. This method is frequently used as the standard by which other typing methods are compared.

These early typing methods were ostensibly developed to understand the epidemiology of *C. difficile* infection at a local level. Many of these investigations found evidence that a single type was responsible for a number of cases within their hospital, thus confirming that *C. difficile* disease could be a cross-infection problem. It soon became apparent, however, that whilst these methods were adequate for local use, there was a need for typing schemes that could be applied to further our understanding of the epidemiology of *C. difficile* disease on a wider scale. To facilitate this, comparisons between typing schemes were performed, and Mulligan et al. [7] found good correlation between the types recognised by plasmid profiling, serotyping, PAGE of cell surface antigens and immunoblotting. Sodium dodecyl sulfate (SDS)-PAGE of whole-cell proteins was applied to 79 isolates in an outbreak investigation and this method yielded a maximum of approximately 40 bands ranging in size from 18 to 100 kilo-daltons (kDa). This investigation showed 60 of the 79 isolates to be indistinguishable. SDS-PAGE of EDTA-extracted cell surface antigens was compared to serogrouping by Ogunsola et al. [8], analysing 61 isolates. This method yielded bands of between 30 and 67 kDa and split their 79 isolates into 17 groups, which generally correlated well with the results of serogrouping, and could in fact, differentiate between some members of the same serogroup. The whole-cell fingerprinting method of pyrolysis mass spectrometry (PMS) has been successfully used as a means of investigating putative *C. difficile* outbreaks [9]. This method has the advantage that it can cope with a large throughput of strains and has a high degree of discrimination. Its disadvantages, however, are the initial cost of the equipment and its inability to assign a permanent type to a strain.

MOLECULAR TYPING METHODS

Molecular typing methods are generally regarded as superior to phenotypic methods in terms of the stability of marker expression and providing greater levels of typeability, and a number of molecular methods have been applied to *C. difficile*. Plasmid profiling proved largely unsuccessful due to the sparse distribution of these extra-chromosomal genetic elements within the species. However, analysis of chromosomal DNA of *C. difficile* was tried by Kuijper et al. [10] who used whole cell DNA restriction endonuclease analysis (REA) using *Hind*III in an

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